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PHYSIOLOGICAL REVIEWS

VOL. 20 APRIL, 1940 No. 2

EXPERIMENTAL HYPERTENSION

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From a historical viewpoint, it is of interest that approximately 100 years separated each of the following observations or discoveries: 1, the circulation of the blood by Harvey; 2, the direct measurement of the blood pressure by Hales; 3, the association of chronic renal disease and hypertrophy of the heart (hypertension assumed) by Bright, and 4, methods for producing a persistent elevation in the blood pressure of experimental animals. It was only 40 years ago that an entirely satisfactory method for determining the arterial blood pressure in man was devised. Just as a careful study of hypertension in man was delayed by the absence of a good method for determining the blood pressure, so have those interested in the problem from the experimental viewpoint been handicapped by lack of simple and accurate means of measuring the blood pressure in unanesthetized animals. The method of Hales, in which the height to which the blood would rise in a tube was determined following direct cannulation of the artery, was improved by employment of the mercury manometer (Poiseuille). The number of determinations which can be carried out in one animal by this method is quite limited. A number of investigators, including Allen (1), have determined the systolic and diastolic pressures in dogs by a slight modification of the auscultatory method as used in patients. This has been unsatisfactory in the hands of most observers, probably due to the uneven contour of the extremities of dogs. A special cuff for use in dogs has been described by Ferris and Hynes (2). The van Leersum (3) loop method in which the carotid artery is placed in a tube of skin is considered satisfactory by many but others have found that it gives very variable results. The Kolls-Cash (4) sphygomanometer allows one to determine both the systolic and diastolic pressures in dogs but it is difficult for one to master the technique. The same may be said for the manometric method of Hamilton, Brewer and Brotman (5)

which is particularly valuable for the measurement of the pressure in small unanesthetized animals. A plethysmographic method for determining the systolic blood pressure of rats was devised by Byrom and Wilson (6), (general anesthesia required), and by Williams, Harrison and Grollman (7), (warming of the animal but no anesthetic agent). This method may be suitable for use on other animals. Although it gives neither systolic nor diastolic levels but rather a figure which corresponds closely to the mean blood pressure, the direct puncture of an artery with a needle connected to a mercury manometer is probably the easiest and is one of the most reliable means of making repeated determinations of the blood pressure in unanesthetized trained dogs. Other methods, too many to enumerate, include the indirect ones of Griffith (8, 9) and of Grant and Rothschild (10). These are particularly adapted to use on small animals.

This review will deal only with experimental hypertension in which the evidence indicates that both the systolic and diastolic pressures are elevated. From the clinical side, it is known that a purely systolic elevation of blood pressure may be associated with aortic insufficiency, hyperthyroidism, arteriosclerosis of the aorta, heart block and arteriovenous fistulae. Some of these conditions can be produced in the experimental laboratory but do not represent true instances of hypertension. Consideration will not be given to the acute arterial hypertension that may be produced by agents such as epinephrine, paredrinol and other chemicals. Although the findings will not be given in detail, it should at least be mentioned that Appelrot (11) and subsequently Handovsky and Goormaghtigh (12, 13, 14) have found that the oral administration to dogs of large doses of "Vitamin D" (vigantol, calciferol) is accompanied by an elevation of blood pressure. Handovsky (14) quotes one experiment in which the pressure remained elevated for the 80 days that the substance was administered and the pressure then gradually declined to the control level. The mechanism responsible for the elevation of pressure has not been determined. Hypertrophy of the media of the arterioles, particularly of the kidney, have been found on histological examination. These alterations in dogs are modified by the removal of the thyroid or the parathyroid glands. Since changes in the arterioles of the kidney are rather marked, it is possible that the hypertension is due to renal ischemia. At any rate, this method is not among the better ones for producing chronic hypertension because the pressure declines if the medication is stopped and, thus far at least, it is not known to have any relationship to hypertension as seen in man.

Successful experimental investigations into the cause of hypertension have in general followed along two lines, one dealing with the elevation of blood pressure which may be produced by one of several procedures carried out on the central nervous system, the other dealing with the hypertension that is renal in origin. If undue space is allotted to the hypertension associated with renal ischemia, it is hoped that it will be excused on the basis of the great interest in and the large amount of work being done on this subject at the present time. For the recent revival of interest in this type of hypertension, Goldblatt is largely responsible. The types that are generally considered to be non-renal in origin will be considered first.

EXPERIMENTAL HYPERTENSION AND THE NERVOUS SYSTEM. I. *Carotid sinus denervation and aortic depressor nerve section.* Following the observations of Cyon and Ludwig on the depressor nerve and of Hering on the carotid sinus, it was shown by Koch and Mies (15) that hypertension can be produced experimentally by bilateral denervation of the carotid sinus and section of the aortic depressor nerve. These observations have been confirmed and elaborated upon by Heymans (16, 17) and by others. Interruption of the afferent nerve impulses which normally buffer or depress the activity of the circulatory centers in the medulla probably permits the vasoconstrictor and cardio-accelerator centers to exert greater effects on the blood pressure. Heymans (18) has observed arterial hypertension maintained at 250 to 300 mm. Hg for periods ranging from 9 to 26 months after section of the moderator nerves; and Nowak and Walker (19) have found that the elevation of pressure may persist for three years. Section of the cardio-aortic and carotid sinus nerves produced only a temporary rise in pressure in some of the dogs in which this procedure has been performed. Heymans (18) states that this is due to the presence of accessory fibers of the cardio-aortic nerves in the vagus or to the taking over of the functions of the moderator nerves by pulmonary and intestinal presso-sensitive nerves. The authors do not give in their publications the percentage of cases in which a prolonged elevation of pressure followed section of the moderator nerves. In a personal communication, Nowak states that at the outset he was successful in producing hypertension in only 30 to 50 per cent of dogs but that more recently hypertension has resulted in 90 per cent of cases. The operative procedure consists of excision of the carotid artery bifurcation (including the carotid sinus) and of the upper cervical portion of the aortic-depressor nerve. Using a slight modification of the procedure used by Heymans, it was found by Green, Degroat and McDonald

(20) that rabbits had only a transient elevation of blood pressure and that dogs had an unstable pressure with usually some elevation which tended to become stabilized subsequently at a level not greatly above the normal. The contradictory results are explained by Heymans as due to the difference in the operative procedure.

Heymans (18) found in dogs that removal of the paravertebral sympathetic ganglia and chain, from the stellate to the pelvic ganglion, prevents or causes the disappearance of this type of hypertension. On the other hand, Nowak and Walker (19) state that preliminary total sympathectomy fails to prevent a moderate rise in blood pressure when the moderator nerves are divided. Further, they found that bilateral splanchnicectomy causes only a temporary fall in existing hypertension produced in this manner and that sympathectomy has to be complete in order to abolish it. Heymans and Bouckaert (21) found that the blood of dogs rendered hypertensive by section of the moderator nerves possesses a higher degree of vasopressor activity than the blood of normal dogs. Braun and Samet (22, 23) state that denervation of the kidneys prevents the development of or abolishes the hypertension associated with section of the moderator nerves. Elaut (24) could not confirm these observations. The hypertension is unaffected by bilateral subtotal adrenalectomy and section of the splanchnic nerves (25).

Descriptions of the histological picture of kidneys of animals with this type of hypertension are contradictory. Nordmann (26) observed no lesions of the arteries or arterioles of the kidney and found only slight glomerular lesions. Goormaghtigh (27), on the contrary, found in hypertensive rabbits, hyperplastic and degenerative lesions of the renal arterioles in addition to lesions of the glomeruli and tubules. He states that the vascular lesions are secondary to the high blood pressure. Hoerner, Fontaine and Mandel (28) stated recently that there is no alteration in the function or histological picture of kidneys of dogs which had had hypertension for two years following section of the moderator nerves. The use of different animals may explain in part at least the contradictory results. Spontaneous arteriosclerotic lesions are not uncommon in rabbits.

All seem to be agreed that section of the moderator nerves results in an elevation of blood pressure but only Heymans and Nowak have obtained with fair consistency a persistent elevation. Insufficient figures are given to allow one to speak with certainty. One gains the impression that section of the moderator nerves usually results in a temporary rise of blood pressure and infrequently in a sustained elevation. The

results, although slightly contradictory, seem to indicate that the elevation of pressure does not result in histological alterations in the arterioles throughout the body. This type of hypertension differs from that due to renal ischemia in that there are greater fluctuations in blood pressure; the elevation is not as apt to be sustained and there are fewer pathological changes in the blood vessels.

The question arises as to whether this type of experimental hypertension induced by removal of the moderator nerves is related in any manner to hypertension as observed in patients. If there is a relationship, it is with essential hypertension rather than the nephropathic variety. It is known that increased vasoconstriction is present in essential hypertension and in experimental hypertension due to removal of the moderator nerves. However, it does not follow necessarily that essential hypertension is due to an abnormality of the carotid-sinus and aortic-depressor nerve mechanisms. As Nowak and Walker (19) have stated, the similarity lies in the end result rather than the cause. It is of interest that experimental hypertension due to removal of the moderator nerves and essential hypertension in man are usually affected very little by division of the splanchnic nerves.

II. *Increased intracranial pressure.* According to Cushing (29), an acute increase in intracranial pressure produces hypertension by causing cerebral anemia. Raab (30) has shown that central anemia stimulates the vasopressor centers and increases their sensitivity to carbon dioxide. Perfusion of the vasomotor center with acids causes a rise in blood pressure and perfusion with alkaline solutions causes a fall. Dixon and Heller (31) found that hypertension of months' or years' duration may be produced in dogs by the injection of kaolin into the cerebrospinal system, thereby causing an increase in intracranial pressure. In several of the animals the blood pressure fell rather abruptly after being elevated for three months. Griffith, Jeffers and Lindauer (32) produced hypertension in rats by the same method. In addition to an increase in the cerebrospinal fluid pressure (33), an elevation of both the systolic and the mean arterial pressure and of the capillary pressure were noted (34). Griffith and Roberts (34) found that an elevation of pressure usually did not occur before the fifth day following the injection of kaolin. In only the occasional rat did the hypertension persist for as long as two months, and the elevation was not permanent in any of them. Lindauer and Griffith (35) were unsuccessful in producing hypertension in cats by this method.

It has been found that this type of hypertension persists in both dogs

(36) and rats (34) following bilateral adrenalectomy if salt and a nonpressor extract of the adrenal cortex are administered. Braun and Samet (37) state that an elevation of pressure can be prevented or reduced to normal by renal denervation. They observed that denervation of one kidney resulted in a decline of the blood pressure, removal of the denervated kidney was followed by a return of the pressure to the hypertensive level. On the other hand, the blood pressure remained depressed if the normal rather than the denervated kidney were removed. Freeman and Jeffers (38) stated recently that sympathetic cardiac innervation, neural or humoral, is necessary for the full development of hypertension produced by increased intracranial pressure. Extensive sympathectomy extending from the fifth thoracic to the fifth lumbar did not prevent the elevation of blood pressure. Pick (39) found that the injection of a relatively small quantity of blood from a dog with hypertension due to kaolin into a normal dog or into a dog in which the kidneys had been denervated resulted in a marked elevation of pressure in the recipient for a number of hours. Injection of blood from a normal dog into a dog with kaolin hypertension had no influence on the blood pressure, but strangely enough, injection of blood from a dog with denervated kidneys into a dog with hypertension due to kaolin resulted in a decline of the blood pressure. Vogt (40) demonstrated, by perfusion experiments, pressor properties in the blood of animals with hypertension due to kaolin. No constricting effect was observed if an adrenalectomy had been performed on the donor of the blood.

The findings by autopsy of the dogs of Dixon and Heller (31) were essentially normal except for dilatation of the cerebral ventricles. The only abnormality noted in the kidneys was that the capsule was unusually adherent. One of these animals had had hypertension for 27 months. Hamperl and Heller (41) noted no gross or microscopic abnormalities in the aorta and in the arterioles of various organs of dogs which had had hypertension for eight months.

Despite the optimistic reports of some observers, one gets the impression that this type of hypertension cannot be produced with great regularity and that the elevation of pressure is usually not persistent. That the elevation of pressure is due to cerebral anemia seems to be fairly well established. Further histological studies of the arterioles of the occasional animal in which the hypertension persists for a year or longer should be performed.

III. *Cerebral anemia.* Nowak and Samaan (42) showed that acute anemia of the cerebral circulation alone causes a marked rise of the

general arterial blood pressure. They perfused the isolated head connected to the body only by the spinal vasomotor pathways. Blalock and Levy (43) state that a sustained elevation of the blood pressure does not usually follow the complete occlusion of the two common carotid and the two vertebral arteries. However, Nowak (19) has found that progressive occlusion of the various cerebral arteries may cause chronic hypertension. The vessels which are ligated include both the external and internal carotids, both vertebral arteries and the anterior spinal arteries. Due to the development of collateral circulation, these procedures do not always result in hypertension. Nowak (44) has recently modified his procedure to include ligation of both subclavian arteries distal to the origin of the common carotid and proximal to the origin of the vertebral artery. This hypertension whether of short or long duration is said to be maintained despite the buffering action of the carotid sinus and aortic depressor nerve mechanism.

The findings of Nowak have not been published in detail, only one blood pressure chart being given, and it would seem very doubtful if this method will prove to be a consistent one for producing hypertension. The elevation of pressure is due almost certainly to cerebral ischemia, and it seems likely that the pressure will decline in most instances as the collateral circulation to the brain increases. Therefore, the available evidence indicates that this is not a consistent method for producing chronic hypertension.

EXPERIMENTAL HYPERTENSION AND THE KIDNEYS. There has been much speculation and controversy for many years as to whether or not diseases of the kidneys are a major factor in the pathogenesis of hypertension in man. There is clinical evidence that the kidneys are diseased in many patients with hypertension. Some pathologists have stated that diseased kidneys are found at autopsy in all patients who have had hypertension for a long time. Fishberg (45) lists the following clinical conditions in which disease of the kidneys or intrarenal blood vessels may lead to hypertension: 1, polycystic disease; 2, chronic pyelonephritis, sometimes unilateral; 3, renal amyloidosis; 4, mercury poisoning; 5, periarteritis nodosa; 6, other forms of renal arterial occlusion; 7, renal hypoplasia; 8, tumors of the kidney; 9, embolism of the renal artery, and finally and probably most important, 10, glomerulonephritis. It has been known for many years that obstruction to the flow of urine may result in hypertension. The greatest interest in recent years has been centered on attempts to find the etiology of essential hypertension which has been defined as a persistently elevated blood pressure of un-

known cause. The central problem has been that of finding the cause for the generalized vasoconstriction, and prior to the work of Goldblatt and associates (46), the earlier theories had been abandoned in the main and the kidney was not seriously considered as the most likely source of this effect. Vascular disease of the kidney itself was considered by most observers to be simply a part of a generalized disease process. Because of the experimental and possible clinical importance of the work of Goldblatt and of those who have used his method, the results will be considered in moderate detail. Other observations which may be of great importance include those of Masugi on the experimental production of glomerulonephritis.

Goldblatt (47) and Fishberg (45) have presented excellent summaries of the experimental evidence for the view that the kidney may play a major part in the development of hypertension. Their points of view will be reëmphasized and reference will be made to some of the more recent work.

I. *Experimental glomerulonephritis.* The early lesions in acute diffuse glomerulonephritis consist of a general swelling and proliferation of the endothelial cells of the glomerular capillaries together with an accumulation of inflammatory exudate within the loops. These changes result in ischemia of the glomeruli and in this one respect there is a similarity between acute glomerulonephritis in the human and hypertension in animals resulting from renal ischemia as produced by the Goldblatt (46) method. The prevailing impression is that the glomerular lesions of diffuse clinical glomerulonephritis are not due to direct invasion of the kidneys by microörganisms but rather to injury by the toxic products of organisms. Diffuse glomerulonephritis complicating various infections usually occurs not during the most active stage of the infection but during convalescence. This fact together with observations on serum sickness and other allergic phenomena suggested to Schick (48) and to von Pirquet (49) that the processes of immunization with the accompanying hypersensitiveness are in some manner connected with the development of glomerulonephritis following an infection.

The earlier literature dealing with attempts to produce glomerulonephritis was reviewed by MacNider (50) and by Leiter (51) in 1924 and by Longcope (52) in 1929. Leiter (51) stated, "Chronic glomerulonephritis has not been produced constantly or even frequently in an experimental animal. . . . Whatever changes were observed in the kidneys could not be interpreted as those of chronic glomerulonephritis."

Longcope (53) was the first to attempt to produce glomerulonephritis in sensitized animals. He produced renal lesions including glomerular changes by injecting repeatedly into various animals horse serum and egg-white. Duval and Hibbard (54) produced what was termed glomerulonephritis by the injection of the endotoxic principle of the streptococcus scarlatinae. The endotoxin was obtained from the viable scarlatinal cultures through the medium of the peritoneal cavity of the rabbit immunized against the homologous strain. Long and Finner (55) observed diffuse renal inflammation following the injection of tuberculin into the kidneys of swine made sensitive to this substance by the presence of mild tuberculosis. Bell and Clawson (56) produced a form of chronic diffuse glomerulonephritis in a monkey by repeated intravenous injections of streptococci over a period of four years. The blood pressure was not determined. Lukens and Longcope (57) produced both focal and diffuse glomerulitis in rabbits by the injection directly into the renal artery of heat killed hemolytic streptococci. Glomerulitis was observed much more frequently in rabbits in which an acute localized streptococcus infection had been produced previously by the intracutaneous injection of living hemolytic streptococci. Somewhat similar lesions were produced by McLeod and Finney (58) using suspensions of streptococcus viridans and by Blackman, Brown and Rake (59) employing an autolysate of type I pneumococcus. These and many other references which cannot be mentioned due to lack of space demonstrate the partial success that has followed the use of methods based on immunity reactions in attempts to produce glomerulonephritis.

It is rather generally agreed that the alterations in the kidneys produced by the methods referred to are not identical with the histological picture of glomerulonephritis as observed in patients. However, this aim has been approached more closely by Masugi (60, 61, 62), who produced what appears to be glomerulonephritis by the injection of anti-kidney serum. Anti-kidney serum was first used by Lindemann (63) in 1900 but it is to Masugi that credit is due for recent interest in this method. He found that by giving rabbits a series of parenteral injections of a suspension of rats' kidneys, the serum of the rabbits developed the property of producing glomerulonephritis when injected into rats. Similar results were obtained in a repetition of this work on rabbits in which ducks were used as the donor of the anti-serum. These findings have been confirmed by Arnott, Kellar and Matthew (64). Smadel (65) found that the anti-kidney sera contain a number of anti-

bodies capable of inducing a severe anaphylactoid reaction and also a nephrotoxic agent that affects the kidney primarily. The nephrotoxic effect is characterized by severe persistent albuminuria with casts and transient anasarca. Hematuria is observed when a severe anaphylactoid reaction is superimposed on the nephrotoxic injury. The nephrotoxic action of anti-kidney serum is removed by absorption with kidney cells or fat-free kidney tissue. Swift and Smadel (66) were able to prevent renal damage due to anti-rat-kidney serum by injecting previously a saline extract of perfused rat kidney. Smadel (67) found that the urinary abnormalities which developed after the injection of nephrotoxin continued until the animal died or was sacrificed. The early renal lesions changed into scarring of the glomeruli and tubules. Studies which were made 3 to 11 months after administration of nephrotoxin revealed chronic progressive glomerulonephritis and generalized vascular lesions. Moderate elevations of blood pressure after treatment with anti-kidney serum have been recorded by Masugi, by Arnott, Kellar and Matthew, by Smadel and Farr (68) and by others. Smadel and Farr state that an elevated blood pressure occurs only in those rats which develop a chronic progressive nephritis and that the hypertension may be influenced by dietary means.

It appears that Masugi and others who have used his method have produced a condition which resembles human glomerulonephritis very closely. However, it is not proven that the pathogenesis of the two is the same. The main point of interest lies in the demonstration that antibodies may be produced which damage the kidneys in a manner similar to that observed in human glomerulonephritis. This is particularly important in view of the belief that sensitization to bacterial infections plays a part in the causation of this disease in man.

II. *Bilateral nephrectomy.* As a preliminary to the following considerations, it should be emphasized that bilateral nephrectomy (69–76) does not result in an elevation of the blood pressure. It was shown by Cash (72) and confirmed by Blalock and Levy (76) that marked interference with the arterial supply to the kidneys results in a significant elevation of blood pressure, provided the entire arterial circulation, including that through the main artery, the smaller vessels in the pedicle, those in the ureter and the capsular vessels, is not completely occluded. In other words, complete absence of the kidneys does not cause a blood pressure elevation, whereas a marked reduction in the renal blood supply causes at least a temporary elevation of blood pressure.

III. *Obstruction to the flow of urine.* The first significant experiments

of this type were those performed by Rautenberg (77) in which temporary occlusion of one of the ureters of rabbits was produced. Following release of the occlusion and removal of the opposite kidney, a moderate elevation of blood pressure was observed. Hartwich (73, 74) and Harrison, Mason, Resnik and Rainey (75) found a moderate elevation of blood pressure following the complete occlusion of both ureters. It was found by Blalock and Levy (76) that the blood pressure returned to normal in approximately six hours following the removal of a single hydronephrotic kidney. Levy, Mason, Harrison and Blalock (78) found that early hydronephrosis is associated with a decrease in renal blood flow, little if any alteration in the arteriovenous oxygen difference, and a decrease in the oxygen consumption. The findings are similar to those encountered in renal ischemia (79) as produced by the Goldblatt method and it is likely that the mechanism of the production of the hypertension associated with each of these conditions is the same. The establishment of a uretero-venous anastomosis, thereby allowing the urine from a normal kidney to enter the venous system, does not result in hypertension (80). Denervation of the kidneys (81, 82) does not abolish the hypertension associated with hydronephrosis. Harrison and associates (83) found that the hydronephrotic kidney generally contains more pressor substances than its unobstructed mate. Williams, Wegria and Harrison (84) state that rats with spontaneous bilateral hydronephrosis have a marked elevation of blood pressure. Extracts of kidneys of rats with induced or spontaneous hydronephrosis cause a greater rise of blood pressure on injection into normal rats than do extracts of the kidneys of normal rats. Furthermore, the sensitivity of hydronephrotic rats to the renal pressor substance is somewhat greater than that of normal animals.

IV. *Subtotal nephrectomy by various methods.* The earlier work on partial nephrectomy includes that of Grawitz and Israel (85), Tuffier (86) and Bradford (87). The first evidence of hypertension associated with cardiac hypertrophy as a result of partial nephrectomy was presented in 1905 by Pässler and Heinecke (88). Many similar experiments have been performed since that time in which the amount of functioning renal tissue has been reduced by simple excision in multiple operations, by ligation of branches of renal arteries, or by nephrectomy combined with one of these procedures. Using one or more of these methods, arterial hypertension was noted in the chronic state by Janeway (89), Allen, Scharf and Lundin (71), Mark and Geisendorfer (90), Chanutin and Ferris (91), Chanutin and Barksdale (92), Wood and

Ethridge (93), Rytand and Dock (94) and others. A temporary elevation of blood pressure was found by Cash (95), Hartwich (73, 74), Ferris and Hynes (2) and others. Particularly important among these observations are those of Chanutin and associates (91, 92) who produced a marked and sustained elevation of blood pressure in rats by partial nephrectomy, the procedure consisting of polar ligation and excision of renal tissue. It was shown that rats which were deprived of approximately 80 per cent of their renal tissue developed progressive renal lesions, arterial hypertension and cardiac hypertrophy. Polyuria was a striking finding. They state the assumption seems warranted that in partially nephrectomized rats with renal insufficiency, an increase of the blood pressure is necessary to maintain an increased volume of urine in order to excrete metabolites in lower concentration. The particular importance of this method lies in the fact that it is the best one for producing hypertension in very small animals, with the possible exception of that of Drury (96), whereas the procedure of Goldblatt (46) is preferable in larger animals. A possible disadvantage for some types of studies is that the hypertension is probably associated with renal insufficiency. Pässler and Heinecke (88) and Chanutin and Ferris (91) noted a failure in producing hypertension in animals becoming cachectic shortly after or during the course of repeated operations.

Wood and Cash (97) observed an elevation of the diastolic as well as the systolic pressure in dogs following subtotal nephrectomy and state that the rise of pressure is not proportionate to the degree of renal insufficiency. Focal accumulations of fat within the media of smaller arteries and arterioles, frequently leading to a marked diminution in the size of the lumen, were found in rats following partial nephrectomy by Wood and Ethridge (93). It is their opinion that the progressive glomerular and arterial renal lesions may be interpreted as a natural sequence of hypertrophy and degeneration brought about by functional strain. Diaz and Levy (98) observed in hypertensive rats a decline of blood pressure following bilateral adrenalectomy. Destruction of the central nervous system was found by Dock and Rytand (99) to cause an abolition of this type of hypertension in rats. They conclude that if a pressor substance is present in the plasma of rats with renal hypertension, it has no direct vasoconstrictor effect but acts through the vasomotor center. They also found (100) that no significant decrease in blood flow per gram of active kidney tissue takes place as hypertension develops. Dock and Rytand (100) state, "Rats which become

hypertensive several months after subtotal nephrectomy do not have renal ischemia; the flow per gram of renal tissue is 19 per cent less than in rats a few days after subtotal nephrectomy, but the same as in rats with unilateral nephrectomy and without hypertension." These findings of Dock and Rytand which indicate an absence of a vasoconstrictor substance in the blood and a normal blood flow per gram of renal tissue are contrary to findings to be discussed later in hypertension as produced by the Goldblatt method.

V. *Kidney damage by various means.* The attempts to produce hypertension by the injection into the renal arteries of liquid paraffin (101), of insoluble Berlin blue (95), and of particles of charcoal (102) have been unsuccessful. Maegraith and McLean (103) reported the production of hypertension in rabbits by the injection of a suspension of Kieselguhr white into one renal artery. Further it was noted that renal denervation abolished the hypertension. Cressman and Blalock (104) have attempted without success to repeat these observations on dogs.

It was found by Dominquez (105) that the intoxication caused by uranium, radium, lead and vanadium does not result in a significant alteration of the blood pressure of rabbits. A moderate temporary elevation of blood pressure (106) which is abolished by renal denervation (107) was produced in rabbits by the repeated intravenous injections of sodium oxalate by Arnott and Kellar. These findings were not confirmed by Scarff and McGeorge (108).

Hartman, Bolliger and Doub (109) produced hypertension in dogs by the use of high voltage Roentgen rays. The alterations included extensive destruction of the renal parenchyma with replacement fibrosis and rather diffuse renal endarteritis. Similar histological alterations were observed by O'Hare and associates (110). Page (111) found that hypertension secondary to irradiation of the kidneys is not abolished by renal denervation.

Pedersen and Bell (112, 113) produced hypertension in rabbits by constricting the renal vein with an aluminum band and by placing a membrane around the kidney in order to prevent the development of venous collateral circulation. These findings were confirmed by Menendez (114) on dogs.

Compression of the kidneys by an oncometer resulted in a slight elevation of the blood pressure in acute experiments by Alwens (115). Loesch (116) found a moderate persistent elevation of blood pressure in dogs in which intermittent brief occlusion of the renal arteries, veins and

ureters was produced repeatedly. Page (117) reported recently that arterial hypertension can be produced in dogs by placing cellophane around one or both kidneys. The pressure rises after several weeks and usually remains elevated. Denervation of the kidney does not interfere with the development of hypertension. At autopsy, the kidney is found to be surrounded by a thick shell of scar tissue.

VI. *Constriction of renal arteries (renal ischemia).* a. *Methods of production.* Katzenstein (118) in 1905 noted a slight elevation of the arterial pressure in acute experiments on dogs following partial occlusion of the renal arteries. Halsted (119) partially occluded one of the two renal arteries with an aluminum band but did not determine the blood pressure. In acute experiments on dogs, Bridgman and Hirose (120) found no alteration of the blood pressure following the constriction of one renal artery by the aluminum band of Halsted and ligation of the opposite renal artery. They stated, "A similar study of animals in whom a constricting band had been left for a considerable period around the renal artery, simulating a chronic lesion, would be of interest, but external events prevented our undertaking it, as had been hoped." Drury (121) in 1932 described briefly a method for the production of renal insufficiency. This consisted of constricting by a silk ligature one renal artery of young rabbits. The diameter of the loop placed about the left renal artery was regulated by tying the silk ligature down on a wire of known diameter. When the animal attained its growth, the opposite kidney was removed. Depending upon the amount of constriction of the renal artery as produced by the ligature, he found that any desired degree of renal insufficiency could be produced. The blood pressure was not determined in these experiments. Recently (1938), further studies using this method were reported by Drury (96). Renal insufficiency of any desired degree was produced in rabbits without resulting pathologic changes in the kidney epithelium. A moderate elevation of the blood pressure was observed prior to the removal of the non-ischemic kidney and it became more marked following nephrectomy.

The fundamental work of Goldblatt and his associates (46) on renal hypertension was begun in 1928. Although it had been suggested that renal ischemia might play an important part in the development of human hypertension, there was no good experimental proof for this deduction. With the working hypothesis that ischemia limited to the kidneys might be the initial condition in the pathogenesis of some types of hypertension, Goldblatt, Lynch, Hanzal and Summerville (46) de-

vised a silver clamp with which the degree of constriction of the main renal artery could be varied and controlled. They found that constriction of one renal artery was followed by a moderate rise of blood pressure which usually returned in several weeks to the level of the control period. Marked constriction of both renal arteries resulted in a marked elevation of the systolic blood pressure which was accompanied by a severe disturbance of renal function and uremia. Moderate constriction of both renal arteries resulted usually in a persistent elevation of the systolic blood pressure. Most interesting is the fact that this condition was unaccompanied by signs of decreased renal function. Findings similar to those noted in dogs were found in experiments (122) on monkeys. It was necessary in some instances to increase the constriction in order to maintain an elevation of the blood pressure which had declined as accessory circulation to the kidney developed. Wood and Cash (97) and Goldblatt found that bilateral renal ischemia resulted in a persistent elevation of the diastolic as well as the systolic pressure. Thus the handicap to the study of hypertension which resulted from inability to produce experimentally with great regularity a persistent and marked elevation of pressure in dogs and monkeys such as one encounters in patients seems to have been removed by the ingenious method of Goldblatt. Mason, Evers and Blalock (123) found that hypertension produced in this manner is not accompanied by an alteration in the renal arteriovenous difference in oxygen content. Levy, Light and Blalock (79) measured the renal blood flow and blood pressure distal to the constriction in animals with persistent hypertension and found both of these functions as well as the oxygen consumption to be decreased.

b. *Pathologic findings.* The pathologic findings in animals following the production of renal ischemia have been described by Goldblatt (46, 124), by Wilson and Pickering (125), by Elaut (126) and by Child (127). When the constriction of the renal arteries was made severe at the beginning, the resulting elevation of blood pressure and uremia were associated with the development of fibrinoid and hyaline degeneration and necrosis of arterioles, with petechiae in some of the organs. The lesions are similar to those observed in the acute malignant phase of essential hypertension in man. The acute arteriolar lesions were most marked in the vessels of the intestinal tract. Goldblatt (124) considers the acute arterial lesions to be the results of the combination of the hypertension and the renal insufficiency. On the other hand, if the constriction of the renal arteries was less severe and resulted in

long standing hypertension without demonstrable damage of renal function, the small arteries and arterioles show thickening of the media and sometimes slight hyalinization of the intima, especially in the retinal arterioles. The changes in the kidneys are mainly in the tubules. Perhaps the most interesting point is that renal ischemia and the associated hypertension result in less damage to the renal arterioles than to the arterioles of most other organs with the exception of the lungs. The pressure in the pulmonary vessels is not increased (128). These findings suggest strongly that the increased intravascular pressure is responsible for the alterations in the vessel walls because, as has been stated, the pressure in the intrarenal vessels is prohibited from rising greatly by the constricting action of the clamp. The recent findings of Wilson and Byrom (129) in experiments on rats in which hypertension was produced by occlusion of one renal artery support this viewpoint. Renal vascular lesions were limited to the non-ischemic kidney. In essential hypertension of patients, the main renal arteries are usually not constricted and the most marked damage is found in the arterioles of the kidneys. Wilson and Pickering (125) found that the severity and extent of the experimental lesions bear a fairly close relationship to the degree of hypertension rather than its duration. It is of significance that the most severe lesions observed by Goldblatt have been in animals that have had a fairly long period of hypertension preceding the induction of renal insufficiency caused by further tightening of the clamps. In such animals in addition to the acute changes, thickening of the media with or without hyalinization of the intima of the arterioles was found. Rupture of the aorta with resulting cardiac tamponade occurred in one of the animals of Blalock, Levy and Cressman (130). Keyes and Goldblatt (131) have studied the eyes of dogs and monkeys in which hypertension had been present for more than five years. Changes similar to those seen in man with benign and malignant essential hypertension were observed. Concentric hypertrophy of the left ventricle was found by Elaut (126). Finally, Moritz and Oldt (132) have presented additional histological evidence suggesting that renal arteriosclerosis is the primary lesion in essential hypertension in man.

c. *Mechanism of production.* That ischemia of a kidney or kidneys is responsible for the elevation of pressure has been demonstrated by a number of experimental procedures. As has been stated, bilateral nephrectomy does not result in hypertension. Release of the arterial constriction or removal of the ischemic kidney (47, 76, 133), the other

kidney being normal, causes an elevated pressure to return to normal in approximately six hours (76). The release of bilateral renal clamps also results in a decline of pressure to the control level. Constriction of the arterial supply to a single transplanted kidney results in an elevation of blood pressure and release of the constriction is followed by a decline (76, 134, 135, 136). Further evidence is furnished by the results of experiments in which the aorta is constricted. Rytand (137) and Goldblatt, Kahn and Hanzal (138) found that constriction of the abdominal aorta just above the site of origin of both main renal arteries results in hypertension whereas constriction of the aorta below the renal vessels has no significant effect on the blood pressure. Steele (139) recently reported that clamping of the aorta above the orifices of the renal arteries in dogs is followed by an elevation of the diastolic level of arterial pressure in the femoral as well as in the carotid arteries. Longcope and McClintock (140) constricted the coeliac axis and the superior mesenteric artery of dogs and found no significant alteration in the blood pressure. Goldblatt (46) found that constriction of the splenic and femoral arteries did not result in a rise of blood pressure. Blalock and Levy (43), by multiple stage operations, produced complete occlusion of the coeliac axis, the superior and inferior mesenteric arteries. A temporary elevation of blood pressure occurred which in some instances did not quite return to the control level, but the elevation was not marked. Thus it would seem certain that ischemia of the kidneys is responsible for the hypertension under consideration. As stated, there is a decrease in the renal blood flow and arterial pressure distal to the point of constriction (79).

The mechanism whereby renal ischemia results in hypertension has been the subject of intensive investigation in the past few years. The control of the arterial pressure is chiefly dependent upon the peripheral resistance, the cardiac output and the blood volume. Freeman and Page (141) found that the production of hypertension by the Goldblatt method does not cause an increase of the plasma volume and Holman and Page (142) observed no alteration in the cardiac output. There is no evidence for an increase of blood viscosity. It would appear then that the hypertension is due to resistance offered to the flow of blood in the finer divisions of the arteries, the arterioles. As Cannon (143) has stated, there may be three explanations of the hypertensive state of the arterioles; 1, excessive discharge of vasoconstrictor impulses from the central nervous system, such as to induce an abnormal narrowing of the channels; 2, increased sensitiveness of the smooth muscle

of the arterioles to natural stimuli which cause contraction, or 3, pathologic constriction of the small vessels because of direct action upon them of unusual chemical agents. There is very strong evidence that hypertension associated with renal ischemia is not produced through a nervous mechanism. It is not prevented or abolished by denervation of the kidneys (111, 144, 126, 145), by section of the splanchnic nerves and excision of the lower four thoracic sympathetic ganglia (146), by subdiaphragmatic splanchnicectomy and removal of the coeliac and upper lumbar ganglia (76), by section of the anterior spinal nerve roots from the fifth thoracic to the third lumbar (147), and by excision of the entire sympathetic chain in the abdomen and chest including denervation of the heart (141, 148, 145). Inconclusive results were obtained in sympathectomized dogs by Alpert, Alving and Grimson (149). Glenn, Child and Page (150) found that destruction of the spinal cord below the fifth cervical vertebra in dogs with hypertension produces an immediate sharp fall of pressure, which is followed by a rise to above the normal but never to the hypertensive level. Glenn and Lasher (151) state that the production of renal ischemia in dogs in which the spinal cord had been destroyed below the fifth cervical vertebra resulted in hypertension. Finally, as has been stated, constriction of the blood supply of a kidney which has been completely denervated by removing it and transplanting it to another part of the body results in hypertension (76). All of these observations seem to prove that a nervous reflex from the ischemic kidneys is not the mechanism responsible for the initiation of the hypertension. As Cannon (143) has stated, there is the bare possibility that the hypothetical agent might influence primarily prevertebral ganglia such as the coeliac and the inferior mesenteric, and by exciting these isolated groups of cells which constrict the splanchnic vessels, might be the occasion of the hypertensive state.

There is no convincing evidence of increased sensitiveness of the smooth muscle of the arterioles to natural stimuli which cause contraction. Bouckaert, Elaut and Heymans (152) reported an increase of the reflex excitability of the vasoconstrictor mechanisms in dogs with renal hypertension. This finding was not confirmed by Verney and Vogt (145). The latter authors noted that sensitivity of hypertensive dogs to injected adrenalin was usually normal, while that to tyramine was often increased. They do not believe hypertension of renal origin to be due to an increased concentration of tyramine in the blood. Rogoff, Marcus and Wasserman (153) have shown that there is no increase

of epinephrine secretion in hypertension due to renal ischemia. Goldblatt and co-workers (46) found that extirpation of one entire adrenal and the medulla of the opposite one did not prevent the elevation of pressure associated with renal ischemia. It was reported by Goldblatt (47), by Blalock and Levy (76) and by Page (154) that bilateral extirpation of the adrenals prevents the rise of blood pressure or causes it to decline if it is already elevated. It is necessary to leave only a small portion of the adrenal cortex for the blood pressure to rise and remain high. Levy and Blalock (155) found that removal of one adrenal and denervation of the other by transplanting it to the neck does not prevent the development of or abolish renal hypertension. In most of the bilaterally adrenalectomized animals of Goldblatt (47), of Page (154) and of Collins and Wood (156), moderate hypertension developed when adequate supportive and substitution therapy was given. Collins and Wood (156) found that the blood pressure of adrenalectomized dogs with renal ischemia declined but did not reach the average normal level even in periods when no cortical extract was given. Enger, Linder and Sarre (157) found that the production of renal ischemia in adrenalectomized dogs resulted in a moderate temporary elevation of the blood pressure. Fasciolo (158) has shown that grafting the ischemic kidney of a dog with hypertension into a chloralosed nephrectomized dog produces a rise of pressure even if the adrenals have been removed. Finally, Rogoff, Nixon and Stewart (159) have found that this type of experimental hypertension may exist in bilaterally adrenalectomized, untreated dogs. Regardless of whether or not hypertension associated with renal ischemia can be induced in adrenalectomized animals, it seems unlikely that the adrenal is implicated specifically in its etiology other than in the sense that the adrenal cortex is important in the maintenance of the blood pressure in all conditions including the normal. Studies have also been performed on the relationship of other endocrine glands to the development and maintenance of renal hypertension. Page and Sweet (160) and Page (154) found that the effect of removal of the hypophysis is to dampen rather than to abolish the effects of renal ischemia. These findings were confirmed by Enger, Linder and Sarre (157). This type of hypertension is not abolished by total thyroidectomy (161) or removal of the ovaries or testes (154). The prevailing evidence indicates that the endocrine glands are not essential for the development of hypertension associated with renal ischemia but that they are important in determining the degree of response.

Since experimental hypertension associated with renal ischemia is

not due to an increase of blood volume or cardiac output, or dependent upon a decrease of the excretory functions of the kidneys, since it is not produced through a nervous mechanism and since the evidence indicates that the endocrine hormones are not directly concerned in its pathogenesis, one is more or less reduced to the possibility that renal ischemia results in hypertension through a chemical mechanism, probably through the release into the circulation of a pressor substance. Supporting this view is much evidence such as the fact that occlusion of the renal vein in the presence of constriction of the renal artery, thereby eliminating the possibility of a hypothetical pressor substance entering the circulation, does not result in hypertension (47). Also interesting is the fact that an ischemic kidney may result in at least a temporary elevation of the arterial pressure in the presence of a normal non-ischemic kidney. But perhaps the most convincing evidence for a humoral factor that has been obtained thus far is that which has resulted from excluding other possible mechanisms.

Renal pressor substance. The recent work on experimental hypertension has focused attention upon the description by Tigerstedt and Bergman (162) in 1898 of a renal pressor substance. This was found in saline extracts of fresh rabbits' kidney or in the dry residue obtained after treating rabbits' kidney with alcohol. This substance which was named "renin" was obtained in the main from the cortex of the kidney. It was described as being non-dialysable, stable at 56° but destroyed by boiling, soluble in water and dilute salt solutions, insoluble in acetone and in 50 per cent alcohol. Renin when injected into anesthetized rabbits caused an elevation of blood pressure which lasted as long as 20 minutes. The observations of Tigerstedt and Bergman indicated that the pressor effect of renin is due to a peripheral action and that it is excreted partially through the kidneys. Some of the workers in this field have confirmed the findings of Tigerstedt and Bergmann, others have obtained only depressor effects while the common finding has been that of a combination of pressor and depressor effects. Collip (163) has stated that extracts of other organs may exert pressor effects. Pickering and Prinzmetal (164) state that the pressor action of renin may be reduced or abolished by anesthetics such as urethane, nembutal and ether. Some of the conflicting results may be explainable on this basis. The purest preparation of Bingel and Strauss (165) was obtained from autolysed renal press juice by fractional precipitation with ammonium sulphate. The findings of Bingel and Strauss (165), Hartwich and Hessel (166), Hessel and Maier-

Hüser (167), Pickering and Prinzmetal (164), and of Landis, Montgomery and Sparkman (168) indicate that renin is either a protein or protein-like substance. The findings of Kohlstaedt, Helmer and Page (169) suggest that renin is an enzyme-like substance which is activated by a kinase-like material contained in the protein fraction of plasma and whole blood. Improvements in methods for preparation and purification of renin have been described by Grossman (170) and Helmer and Page (171). The latter authors state that renin prepared by their method is stable for at least two months when kept cold. Landis, Montgomery and Sparkman (168) have found that the pressor activity can be made more consistent by appropriate heating. Furthermore, they made the important observation that peripheral blood flow is not reduced during the period when the blood pressure is elevated. Other pressor substances were studied and the specially treated saline kidney extract was the only one which elevated peripheral blood pressure without simultaneously reducing skin temperature in the ear of the warmed rabbit. This property of renin suggests a mechanism similar to that of hypertension in man but it differs in that the pressor effect of renin is not sustained more than 45 minutes and subsequent repeated injections yield smaller responses. Landis, Jeffers and Shiels (172) have found that heating the extract to 55° precipitates at least some of the pressor substance in addition to diminishing the depressor effects.

Williams, Harrison and Mason (173) showed that the pressor response of renin is not abolished by cocaine as is that of tyramine. Two different pressor substances were obtained from extracts of renal tissue. Williams and Grossman (174) obtained two pressor substances by perfusion of isolated kidneys of hogs and dogs. One of these substances resembled Tigerstedt's renin in its properties while the other was believed to be adrenalin or some adrenalin-like substance. The pressor effect of the latter substance was enhanced by cocaine and diminished by ergotamine. Williams (175) noted subsequently that the rise of blood pressure in white rats produced by renin is enhanced by cocaine and inhibited by ergotamine. Since adrenine is similarly affected, Williams suggested that the prolonged rise of pressure with renin injections is due to gradual liberation of an adrenine-like substance. Hessel (176), Friedman, Abramson and Marx (177) and Helmer and Page (171) have found that the pressor action of renin is not abolished by ergotamine nor potentiated by cocaine. The contradictory results may be due to the use of different test animals or to the presence of

impurities in the extracts. Boylston, McEwen and Ivy (178) were unable to obtain a pressor substance in significant amounts from the ischemic kidneys of hypertensive dogs by perfusing them with Locke's solution. No significant differences in the vasoconstricting properties of normal plasma and of plasma from hypertensive dogs on arterial rings were found by Wakerlin and Yanowitz (179).

Grossman and Williams (180) found that the kidneys of young rats contain a greater quantity of renal pressor substance than those of old rats, and that the blood pressure response of old rats to injection of the renal pressor substance is greater. It was noted by Friedman et al. (181) that ablation of the adrenal glands was followed by a progressive diminution in response to the renal pressor substance but not to other vasoconstrictor substances. The sensitivity to renin returned when cortin was given. Similar results were obtained by Williams, Diaz, Burch and Harrison (182). Also it was noted that the kidneys of adrenalectomized rats usually contain more of the renal pressor substance than do those of normal rats.

Regarding the site of action of the renal pressor substance, Hessel and Maier-Hüser (167) stated that the vessels of the extremities, intestines and kidneys are constricted. Merrill, Williams and Harrison (183) observed that the injection of renin caused a rise of blood pressure after destruction of the spinal cord and after exclusion of the hypophysis, adrenals, pancreas, liver and kidneys from the circulation. The same authors confirmed the observation of Tigerstedt and Bergman (162) that animals which had been subjected to nephrectomy several days previously exhibited a marked increase in sensitivity to renin. Tigerstedt and Bergman ascribed this difference to failure of excretion of renin by the nephrectomized animal. However, Merrill et al. (183) found that this increase in sensitivity did not develop immediately after nephrectomy but appeared one or more days later. This observation led Grollman, Harrison and Williams (184) to suspect that normal renal tissue might form some substance capable of antagonizing the action of renin. A method for the bio-assay of renin in which nephrectomized dogs are used was described by Wakerlin and Chobot (185). Leiter and Eichelberger (186) observed that the injection of renin into dogs with renal ischemia produced a more prolonged pressor response than was found in normal dogs. A vasoconstrictor effect was demonstrated (183) on perfusion of the isolated leg. Similar findings were obtained by Friedman, Abramson and Marx (177). Subsequent experiments by Merrill et al. (187) indicated that the kidney is par-

ticularly sensitive to the action of renin as the diminution of renal blood flow was more pronounced than the elevation of blood pressure. The results of these and other experiments suggested to the authors that renin produces contraction of the efferent glomerular vessels. Similar results were obtained by Corcoran and Page (188). On the other hand, Steele and Schroeder (189) found that the injection of renin caused an increase of the renal blood flow.

Harrison and his associates (83, 190), Prinzmetal and Friedman (191) and Govaerts and Dicker (192) found that extracts of kidneys of dogs with hypertension exerted greater pressor effects than those of control kidneys. Comparison of the extracts prepared from the two kidneys of dogs rendered hypertensive by compression of one renal artery showed a greater pressor effect from the abnormal than from the normal kidney. Increased pressor properties in the blood of dogs with renal ischemia and hypertension could not be demonstrated by Prinzmetal, Friedman and Rosenthal (193), by Page (194), by Collins and Hoffbauer (195) and by Heymans and Bouckaert (21). Dicker (196) states that the blood of hypertensive dogs contains pressor substances which are absent in normal dogs. This observation would appear to need confirmation. Houssay and Taquini (197, 198, 199) made comparative studies of the vasoconstrictor action of the plasma of venous blood from both normal and ischemic kidneys, the Laewen-Trendelenburg method on the toad being used. They found in all instances that the venous blood from an ischemic kidney causes greater vasoconstriction than that from a normal kidney or any other organ. Further, it was noted that the venous blood from the normal kidney of an animal with unilateral renal ischemia contains less of the pressor substance than the arterial blood, indicating that the normal kidney destroys this substance. Mason (200) has been unable to confirm these observations on North American toads and bull frogs using the method employed by Houssay and Taquini. In connection with this general problem, it is of interest that Verney and Vogt (145) found in experiments in which an isolated kidney and a loop of small intestine were each perfused by a heart-lung preparation that the perfused kidney liberates a substance which produces vasoconstriction in the gut. Wakerlin and Chobot (201) state that their experiments yield no evidence for the possible rôle of renin in the maintenance of normal blood pressure.

Although there is no conclusive proof of the existence of a known or new pressor substance in the blood, spinal fluid or urine in experi-

mental hypertension due to renal ischemia, the findings suggest that the increase of blood pressure is dependent on the excessive formation of a pressor agent which is also present in normal kidneys. As Harrison (190) has stated, other possibilities are that the substance formed in the kidney with a defective blood supply might be somewhat different in composition and more active pharmacologically, that the kidney with an impaired circulation is less capable of excreting a normal quantity of pressor substance, that the pressor agent may be some intermediary metabolic product and the reaction fails to go on to completion in the absence of a normal blood supply, and lastly that the occurrence of increased pressor activity in the ischemic kidney may bear no relationship to the rise of blood pressure. A further remote possibility is that there is a decrease of depressor substances rather than an increase of the pressor ones.

As has been stated, the hypertension which results from constriction of the blood supply to one kidney only usually does not persist. The fact that it occurs at all indicates that the hypothetical agent is one which the normal kidney cannot excrete rapidly. The fact that the hypertension does not persist indicates that the normal kidney is a factor in determining the response to renal ischemia. Further evidence which supports this was found by Blalock and Levy (76) in experiments in which removal of the non-ischemic kidney resulted in hypertension in animals with unilateral ischemia and a normal blood pressure. Similar results have been obtained by Katz, Mendlowitz and Friedman (202), by Verney and Vogt (145) and by Fasciolo (199). Rodbard (203) states that the destruction, neutralization or elimination of the chemical mediator can be accomplished at a rapid rate only in the presence of kidney tissue. Evidence that the normal kidney is capable of reducing the rise in pressure produced by the ischemic kidney is in keeping with the experiments of Grollman, Harrison and Williams (184) in which it was found that a substance which lowers the blood pressure of hypertensive rats (subtotal nephrectomy) when administered orally can be obtained from the kidneys of hogs. The conception that normal renal tissue produces something capable of lowering a pathological elevation in the blood pressure is not supported by the fact that an occasional dog with only unilateral renal ischemia will have a persistent elevation in the blood pressure. Furthermore, it has been found by Blalock, Levy and Cressman (130) that a high percentage of animals with unilateral renal ischemia combined with intestinal ischemia, produced by the gradual complete occlusion of the

coeliac axis, the superior and inferior mesenteric arteries, will have a sustained elevation of blood pressure.

Attempts to modify the hypertension (renal ischemia). Many attempts to influence the hypertension associated with renal ischemia have been made in addition to those described already in connection with operations on the nervous system and the endocrine glands. Cash and Wood (204) found that diets which cause a marked gain in weight in dogs with renal hypertension result in a marked elevation of the systolic blood pressure. Reduction of weight in these animals was associated with a decline of the systolic but not of the diastolic pressure. Verney and Vogt (145) found that increased dietary loads elicited a reversible rise of the blood pressure level of hypertensive dogs with renal ischemia, sodium chloride being more effective in this respect than urea or meat. They state that the sensitivity of the arterial pressure to changes in dietary load is dependent upon renal ischemia and not upon a simple reduction in the quantity of functioning renal tissue. Particularly interesting are the preliminary observations of Dill and Erickson (205) that an eclampsia-like syndrome occurs in pregnant dogs and rabbits when the renal arteries are constricted. On the other hand, unpublished observations by Goldblatt, by Grollman, Harrison and Williams and by Dawson, Cressman and Blalock show that there is a decline in the pressure in hypertensive dogs and rats during the terminal part of the pregnancy period.

Attempts to prevent the development of or to abolish hypertension associated with renal ischemia have in the main been unsuccessful. The passage of the venous blood returning from the kidneys through the liver (206, 207) does not affect this type of hypertension. Previous damage to the kidneys, partial constriction of the renal veins and hyperpyrexia are without influence (207). Levy and Blalock (207) noted that a severe illness such as distemper is usually accompanied by a decline of the elevated blood pressure, which is another point in common with hypertension as observed in man. Preliminary experiments by Davis and Barker (208) indicate that the decline of blood pressure following the giving of cyanates to dogs with renal ischemia is usually greater if a splanchnicectomy has been performed. As Goldblatt (209) has stated, one obvious procedure which suggests itself in clinical or experimental hypertension is the possible improvement of blood supply to the functioning components of the kidney by attempts to increase the collateral circulation. He stated, "If, before constricting the renal artery, the kidney is decapsulated and

adipose tissue or muscle is attached to the denuded cortical surface, the accessory circulation becomes very prominent and interferes with the development of pronounced elevation in blood pressure." MacNider and Donnelly (210) and Davis and Tullis (211) have reported results which indicate that an accessory blood supply to the normal kidney may be created artificially. Mansfield, Weeks, Steiner and Victor (212) found that the hypertension produced by constriction of the renal arteries is lowered by pexis between the kidney and the omentum or spleen. It was observed that the reduction in blood pressure with the omental union was only temporary whereas that with the kidney-splenic union was sustained. It is difficult to draw conclusions from such experiments, as is demonstrated by the fact that animals may survive complete occlusion of both main renal arteries (47, 76) when it is effected gradually without any attempt being made to create a new collateral blood supply. Further, the employment of such a procedure in the treatment of human hypertension would be rarely indicated because the vascular disease most frequently includes involvement of the preglomerular arteries. In the instances of sclerosis of the larger renal vessels, an attempt to cause an increase of the collateral blood supply might result in benefit, but even this is very doubtful. The results (213) of nephro-omentopexy in man are not very encouraging. Unfortunately, no means has as yet been devised to make a kidney survive following homo-transplantation.

SUMMARY (RENAL ISCHEMIA)

In concluding this consideration, it may be stated the evidence indicates that diminution in the renal blood flow produces peripheral vasoconstriction and hypertension by means of the action of a pressor substance which is formed in the ischemic kidney and which enters the blood stream. The exact nature of the product elaborated by the kidney has not been identified. It is likely that it is produced in small amounts over a long period of time and it is possible that its identification is not possible by the methods available at present. The most hopeful line of investigation from the therapeutic viewpoint lies in the possibility of finding a specific anti-pressor substance. An interesting application which has already resulted from this work consists of the disappearance of hypertension following nephrectomy in patients with unilateral pyelonephritis and vascular disease (214–218).

It is not believed that renal ischemia is responsible for all instances of experimental hypertension. Katz (202) is of the opinion that hyper-

tension of renal origin depends on the ratio of ischemic to normal kidney tissue rather than on the amount of ischemic renal tissue alone. Some believe that ischemia is only one of many abnormalities that may result in hypertension. However, it is interesting that the renal blood flow is known to be reduced in hypertension produced by the Goldblatt method and in that due to ureteral occlusion; it is almost certainly reduced in coarctation of the aorta and in the chronic form of experimental glomerulonephritis and the total renal blood flow is diminished in hypertension due to a reduction in the amount of functioning renal tissue as accomplished by various methods even though the evidence indicates that it is not reduced per gram of kidney tissue; it may possibly be reduced in hypertension due to the injection of kaolin into the cerebrospinal system and in that due to section of the moderator nerves, the evidence for this being contradictory, some claiming that denervation of the kidneys abolishes the hypertension. Many of the observations that have been enumerated probably have a distinct bearing on clinical hypertension. Karsner (219) stated recently, "The observations in experimental animals and in man give no support to the view that essential hypertension is different from renal hypertension. . . . Failure to demonstrate any signs of renal incompetency in life is not indicative of the absence of arteriolar disease of the kidneys. . . . The burden of proof must be with those who make a qualitative classification of forms of chronic hypertension in opposition to the view here expressed that the disease is basically unitary."

Granting that there are some types of hypertension that are non-renal in origin, the evidence which has been reviewed indicates that most instances of experimental and probably of clinical hypertension are related to some abnormality in function of the kidneys.

ADDENDA

A number of important articles on experimental hypertension have appeared since the completion of this review. No attempt will be made to refer to all of them. Particularly outstanding among the recent contributions are the following.

1. Goormaghtigh and Grimson (220) have presented evidence in support of the existence of an endocrine gland in the wall of the renal arterioli and are of the opinion that it is principally the afibrillary muscle cells of the media of the arterioli which are the endocrine elements. Goormaghtigh (221) states, "Renal ischemia causes hypertrophy and multiplication of certain cells of the media of the renal

arterioli which normally lack myofibrillae and which in the rabbit possess the cytologic characteristic of endocrine cells. Provided it is fairly pronounced it causes the transformation of muscle cells with myofibrillae into afibrillary cells which always become granular in the rabbit and sometimes, though more discretely so, in the dog. If ischemia lasts for several months many afibrillary renal arteriolar elements in the dog become granular and acquire the cytologic endocrine characteristics seen in the rabbit." The most obvious objection to this interpretation is that the arterioli of all organs are said to contain afibrillary cells and it is known that progressive constriction of the arterial supply of organs other than the kidneys does not result in hypertension. Goormaghtigh (221) states that this difference is due to the fact that the afibrillary cells of the various organs are not identical and that in no organ except the kidneys is there such close contact between the cells of the arteriolar media and the cells of the parenchyma.

2. Page (222) has recently described the results of a large number of experiments bearing upon the mechanism of renin tachyphylaxis (decreased pressor response to consecutive injections). It has been shown previously that purified renin, when incorporated in Ringer solution and used as a perfusate for isolated organs, exhibited no vasoconstrictor action, presumably because of the absence of a protein activator present in normal blood (169, 223). The pressor action is restored by the addition of partially purified activator, or by addition of blood.

The isolated rabbit's ear exhibits tachyphylaxis to renin, which is abolished by the addition of renin activator, and the phenomenon in the isolated organ appears to be due solely to exhaustion of the supply of activator in the perfusate. In the intact tachyphylactic animal, however, the pressor effect of renin is not restored by addition of activator alone, and Page concludes that the refractory state is the consequence of two phenomena, (a) exhaustion of renin-activator, and (b) the appearance of an anti-pressor substance, or the development of an "anti-pressor state."

The hypothetical anti-pressor substance was not directly demonstrated. As tachyphylaxis was not studied in nephrectomized animals, it is not clear whether this anti-pressor substance is related in any way to that claimed to be found in and formed in kidneys by Grollman, Harrison and Williams (184, 224).

More recently Page and Helmer (225) have studied the interaction of renin and renin activator and state they have isolated the resulting product as the crystalline oxalate and picrate. This substance, desig-

nated "angiotonin" is water soluble, heat-stable, dialyzable, fluorescent, and gives a positive Sakaguchi reaction. It is strongly pressor with a somewhat more prolonged effect than adrenalin, its action being primarily upon the peripheral blood vessels. Only moderate tachyphylaxis is observed following repeated injections into cats and dogs. The authors believe "angiotonin" is an intermediate product of the interaction of renin (enzyme) and renin-activator (substrate) as prolonged contact between the two reduces the yield of pressor substance. It is their view that renin is an enzyme, contained in the kidneys, and without pressor properties, which reacts with activator in blood to form "angiotonin," a pressor substance which may provide the body with a humoral means of regulating arterial pressure.

3. Harrison, Grollman and Williams (226) have found that the blood pressure of dogs with hypertension due to renal ischemia may be reduced by the administration of their renal anti-pressor substance. This extract which also reduces the blood pressure of rats with hypertension due to subtotal nephrectomy does not cause a decline in the blood pressure of normal rats (184, 224). Some of their animals have become seriously ill while under treatment and this finding has delayed study of this form of therapy for patients. Although a few patients have been treated with encouraging results, the authors state that their extract is not yet sufficiently free from impurities to be ready for general clinical trial (226).

4. Recent studies of the respiratory metabolism of tissue slices from normal and hypertensive kidneys have yielded conflicting results (227, 228).

REFERENCES

(1) Allen, F. S. J. Metabol. Research **4**: 431, 1923.
(2) Ferris, H. W. and J. F. Hynes. J. Lab. and Clin. Med. **16**: 597, 1931.
(3) van Leersum, E. C. Arch. **142**: 377, 1911.
(4) Kolls, A. C. and J. R. Cash. Bull. Johns Hopkins Hosp. **34**: 49, 1923.
(5) Hamilton, W. F., G. Brewer and I. Brotman. Am. J. Physiol. **107**: 427, 1934.
(6) Byrom, F. B. and C. Wilson. J. Physiol. **93**: 301, 1938.
(7) Williams, J. R., Jr., T. R. Harrison and A. Grollman. J. Clin. Investigation **18**: 373, 1939.
(8) Griffith, J. Q., Jr. and L. H. Collins, Jr. Am. Heart J. **8**: 671, 1938.
(9) Griffith, J. Q., Jr. Proc. Soc. Exper. Biol. and Med. **32**: 394, 1934.
(10) Grant, R. T. and P. Rothschild. J. Physiol. **81**: 265, 1935.
(11) Appelrot, S. Am. J. Physiol. **105**: 294, 1933.
(12) Handovsky, H. and N. Goormaghtigh. Arch. internat. pharmacod. et therap. **56**: 376, 1937.

(13) GOORMAGHTIGH, N. AND H. HANDOVSKY. Compt. rend. Soc. biol. **118:** 1616, 1935.
(14) HANDOVSKY, H. J. Physiol. **90:** 17, 1937 (Proc.).
(15) KOCH, E. AND H. MIES. Krankheitsforschung **7:** 241, 1929.
(16) HEYMANS, C. AND J. J. BOUCKAERT. Compt. rend. Soc. biol. **106:** 471, 1931.
(17) HEYMANS, C. Compt. rend. Soc. biol. **107:** 1293, 1931.
(18) HEYMANS, C. Surgery **4:** 487, 1938.
(19) NOWAK, G. J. G. AND I. J. WALKER. New England J. Med. **220:** 269, 1939.
(20) GREEN, M. F., A. F. DEGROAT AND C. H. MCDONALD. Am. J. Physiol. **110:** 513, 1935.
(21) HEYMANS, C. and J. J. BOUCKAERT. Proc. Soc. Exper. Biol. and Med. **39:** 94, 1938.
(22) BRAUN, L. AND B. SAMET. Wien. klin. Wchnschr. **47:** 65, 134, 1934.
(23) BRAUN, L. AND B. SAMET. Wien. klin. Wchnschr. **48:** 940, 1935.
(24) ELAUT, L. Compt. rend. Soc. biol. **199:** 318, 1935.
(25) FONTAINE, R. AND P. MANDEL. Compt. rend. Soc. biol. **127:** 448, 1938.
(26) NORDMANN, M. Krankheitsforschung **7:** 268, 1929.
(27) GOORMAGHTIGH, N. Ann. D'anat. Path. **8:** 585, 1931.
(28) HOERNER, G., R. FONTAINE AND P. MANDEL. Arch. mal. coeur **31:** 1090, 1938.
(29) CUSHING, H. Am. J. Med. Sc. **125:** 1017, 1903.
(30) RAAB, W. Arch. Int. Med. **47:** 727, 1931.
(31) DIXON, W. E. AND H. HELLER. Arch. exper. Path. u. Pharmakol. **166:** 265, 1932.
(32) GRIFFITH, J. Q., JR., W. A. JEFFERS AND M. A. LINDAUER. Am. J. Physiol. **113:** 285, 1935.
(33) GRIFFITH, J. Q., JR., W. A. JEFFERS AND M. A. LINDAUER. Am. J. Physiol. **118:** 1, 1937.
(34) GRIFFITH, J. Q., JR. AND E. ROBERTS. Am. J. Physiol. **124:** 86, 1938.
(35) LINDAUER, M. A. AND J. Q. GRIFFITH, Jr. Proc. Soc. Exper. Biol. and Med. **39:** 547, 1938.
(36) JEFFERS, W. A., M. A. LINDAUER AND F. D. W. LUKENS. Proc. Soc. Exper. Biol. and Med. **37:** 260, 1937.
(37) BRAUN, L. AND B. SAMET. Arch. exper. Path. u. Pharmakol. **177:** 662, 1933.
(38) FREEMAN, N. E. AND W. A. JEFFERS. Am. J. Physiol. **126:** P. 493, 1939.
(39) PICK, E. P. Wien. klin. Wchnschr. **48:** 634, 1935.
(40) VOGT, H. Klin. Wchnschr. **17:** 1148, 1938.
(41) HAMPERL, H. AND H. HELLER. Arch. f. exper. Path. **173:** 283, 1933.
(42) NOWAK, S. J. G. AND A. SAMAAN. Arch. internat. pharmacod. et therap. **51:** 463, 1935.
(43) BLALOCK, A. AND S. E. LEVY. Surgery **5:** 175, 1939.
(44) NOWAK, S. J. G. Personal communication, 1939.
(45) FISHBERG, A. M. Hypertension and nephritis. Lea and Febiger, Philadelphia, 1939.
(46) GOLDBLATT, H., J. LYNCH, R. F. HANZAL AND W. W. SUMMERVILLE. J. Exper. Med. **59:** 347, 1934.
(47) GOLDBLATT, H. Ann. Int. Med. **11:** 69, 1937.

(48) Shick, B. In Escherich and Schick: Scharlach. Alfred Holder, Vienna, 1912, p. 151.
(49) von Pirquet, C. E. Arch. Int. Med. **7:** 259, 383, 1911.
(50) MacNider, W. deB. Physiol. Rev. **4:** 595, 1924.
(51) Leiter, L. Arch. Int. Med. **33:** 611, 1924.
(52) Longcope, W. T. Bull. Johns Hopkins Hosp. **45:** 335, 1929.
(53) Longcope, W. T. J. Exper. Med. **18:** 678, 1913.
(54) Duval, C. W. and R. J. Hibbard. J. Exper. Med. **44:** 567, 1926.
(55) Long, E. R. and L. L. Finner. Am. J. Path. **4:** 571, 1928.
(56) Bell, E. T. and B. J. Clawson. Am. J. Path. **7:** 57, 1931.
(57) Lukens, F. D. W. and W. T. Longcope. J. Exper. Med. **53:** 511, 1931.
(58) McLeod, N. and G. G. Finney. Bull. Johns Hopkins Hosp. **51:** 300,1932.
(59) Blackman, S. S., J. H. Brown and G. Rake. Bull. Johns Hopkins Hosp. **48:** 74, 1931.
(60) Masugi, M. Beitr. z. Path. Anat. **91:** 82, 1933.
(61) Masugi, M. Beitr. z. Path. Anat. **92:** 429, 1934.
(62) Masugi, M. and Y. Sato. Virchow's Arch. **293:** 615, 1934.
(63) Lindemann, W. Ann. Inst. Pasteur **14:** 49, 1900.
(64) Arnott, W. M., R. J. Kellar and G. D. Matthew. Edinburgh Med. J. **43:** 233, 1936.
(65) Smadel, J. E. J. Exper. Med. **64:** 921, 1936.
(66) Swift, H. F. and J. E. Smadel. J. Exper. Med. **65:** 557, 1937.
(67) Smadel, J. E. J. Exper. Med. **65:** 541, 1937.
(68) Smadel, J. E. and L. E. Farr. J. Exper. Med. **65:** 527, 1937.
(69) Mosler, E. Ztschr. klin. Med. **74:** 297, 1912.
(70) Backmann, E. L. Ztschr. ges. exper. Med. **4:** 63, 1916.
(71) Allen, F. M., R. Scharf and H. Lundin. J. A. M. A. **85:** 1698, 1925.
(72) Cash, J. R. Proc. Soc. Exper. Biol. and Med. **23:** 609, 1926.
(73) Hartwich, A. Verhandl. deutsch. Ges. inn. Med. **41:** 187, 1929.
(74) Hartwich, A. Ztschr. ges. exper. Med. **69:** 462, 1930.
(75) Harrison, T. R., M. F. Mason, H. Resnik and J. Rainey. Trans. Assn. Am. Physicians **51:** 280, 1936.
(76) Blalock, A. and S. E. Levy. Ann. Surg. **106:** 826, 1937.
(77) Rautenberg, E. Deutsch. med. Wchnschr. **36:** 551, 1910.
(78) Levy, S. E., M. F. Mason, T. R. Harrison and A. Blalock. Surgery **1:** 238, 1937.
(79) Levy, S. E., R. A. Light and A. Blalock. Am. J. Physiol. **122:** 38, 1938.
(80) Geer, W. A. and L. R. Dragstedt. Ann. Surg. **108:** 263, 1938.
(81) Mason, M. F., H. Resnik, A. S. Minot, J. Rainey, C. Pilcher, and T. R. Harrison. Arch. Int. Med. **60:** 312, 1937.
(82) Dicker, E. Compt. rend. Soc. biol. **126:** 910, 1937.
(83) Harrison, T. R., A. Blalock and M. F. Mason. Proc. Soc. Exper. Biol. and Med. **35:** 38, 1936.
(84) Williams, J. R., Jr., R. Wegria and T. R. Harrison. Arch. Int. Med. **62:** 805, 1938.
(85) Grawitz, P. and O. Israel. Virchow's Arch. **77:** 315, 1879.
(86) Tuffier, T. Quoted by R. M. Pearce. J. Exper. Med. **10:** 632, 1908.
(87) Bradford, J. R. J. Physiol. **23:** 415, 1899.
(88) Pässler and Heinecke. Verhandl. deutsch. path. Ges. **9:** 99, 1905.

(89) JANEWAY, T. C. Proc. Soc. Exper. Biol. and Med. **6**: 109, 1909.
(90) MARK, R. E. AND H. GEISENDORFER. Ztschr. ges. exper. Med. **74**: 350, 1930.
(91) CHANUTIN, A. AND E. B. FERRIS. Arch. Int. Med. **49**: 767, 1932.
(92) CHANUTIN, A. AND E. E. BARKSDALE. Arch. Int. Med. **52**: 739, 1933.
(93) WOOD, J. E., JR. AND C. ETHRIDGE. Proc. Soc. Exper. Biol. and Med. **30**: 1039, 1933.
(94) RYTAND, D. A. AND W. DOCK. Arch. Int. Med. **56**: 511, 1935.
(95) CASH, J. R. Bull. Johns Hopkins Hosp. **35**: 168, 1924.
(96) DRURY, D. R. J. Exper. Med. **68**: 693, 1938.
(97) WOOD, J. E. JR. AND J. R. CASH. J. Clin. Investigation **15**: 543, 1936.
(98) DIAZ, J. T. AND S. E. LEVY. Am. J. Physiol. **125**: 586, 1939.
(99) DOCK, W. AND D. A. RYTAND. Proc. Soc. Exper. Biol. and Med. **32**: 374, 1934.
(100) DOCK, W. AND D. A. RYTAND. Proc. Soc. Exper. Biol. and Med. **36**: 196, 1937.
(101) SENATOR, H. Ztschr. klin. Med. **72**: 189, 1911.
(102) APFELBACH, C. W. AND C. R. JENSEN. J. Clin. Investigation **10**: 162, 1931.
(103) MAEGRAITH, B. G. AND F. J. MCLEAN. J. Physiol. **92**: 44, 1938.
(104) CRESSMAN, R. D. AND A. BLALOCK. Proc. Soc. Exper. Biol. and Med. **40**: 258, 1939.
(105) DOMINQUEZ, R. Arch. Path. **5**: 577, 1928.
(106) ARNOTT, W. M. AND R. J. KELLAR. Brit. J. Exper. Path. **16**: 265, 1935.
(107) ARNOTT, W. M. AND R. J. KELLAR. J. Path. and Bact. **42**: 141, 1936.
(108) SCARFF, R. W. AND M. MCGEORGE. Brit. J. Exper. Path. **18**: 59, 1937.
(109) HARTMAN, F. W., A. BOLLIGER AND H. P. DOUB. Am. J. Med. Sc. **172**: 487, 1926.
(110) O'HARE, J. P., H. ALTNOW, T. D. CHRISTIAN, A. W. CALHOUN AND M. C. SOSMAN. Boston M. and S. J. **194**: 43, 1926.
(111) PAGE, I. H. Am. J. Physiol. **112**: 166, 1935.
(112) PEDERSEN, A. H. Arch. Path. and Lab. Med. **3**: 912, 1927.
(113) BELL, E. T. AND A. H. PEDERSEN. Ann. Int. Med. **4**: 227, 1930.
(114) MENENDEZ, E. B. Compt. rend. Soc. biol. **113**: 461, 1933.
(115) ALWENS, W. Deutsch. Arch. klin. Med. **98**: 137, 1910.
(116) LOESCH, J. Zentralbl. inn. Med. **54**: 145, 177, 1933.
(117) PAGE, I. H. Science **89**: 273, 1939.
(118) KATZENSTEIN, M. Virchow's Arch. **182**: 327, 1905.
(119) HALSTED, W. S. J. Exper. Med. **11**: 373, 1909.
(120) BRIDGMAN, E. W. AND K. HIROSE. Arch. Int. Med. **21**: 351, 1918.
(121) DRURY, D. R. Proc. Soc. Exper. Biol. and Med. **29**: 856, 1932.
(122) GOLDBLATT, H. J. Exper. Med. **65**: 671, 1937.
(123) MASON, M. F., R. EVERS AND A. BLALOCK. Proc. Soc. Exper. Biol. and Med. **36**: 819, 1937.
(124) GOLDBLATT, H. J. Exper. Med. **67**: 809, 1938.
(125) WILSON, C. AND G. W. PICKERING. Clin. Sci. **3**: 343, 1938.
(126) ELAUT, C. R. Compt. rend. Soc. biol. **123**: 1244, 1936.
(127) CHILD, C. G. J. Exper. Med. **67**: 521, 1938.
(128) STEINITZ, F. S. AND L. N. KATZ. Am. J. Physiol. **126**: p. 639, 1939.

(129) Wilson, C. and F. B. Byrom. Lancet **236:** 136, 1939.
(130) Blalock, A., S. E. Levy and R. D. Cressman. J. Exper. Med. **69:** 833, 1939.
(131) Keyes, J. E. L. and H. Goldblatt. Arch. Ophth. **20:** 812, 1938.
(132) Moritz, A. R. and M. R. Oldt. Am. J. Path. **13:** 679, 1937.
(133) Dicker, E. Compt. rend. Soc. biol. **125:** 1046, 1937.
(134) Glenn, F., C. G. Child and G. J. Heuer. Ann. Surg. **106:** 848, 1937.
(135) Houssay, B. A. and J. C. Fasciolo. Bol. Acad. Med. Buenos Aires **34:** 342, 1937.
(136) Dicker, E. Compt. rend. Soc. biol. **126:** 912, 1937.
(137) Rytand, D. A. J. Clin. Investigation **17:** 391, 1938.
(138) Goldblatt, H., J. R. Kahn and R. F. Hanzal. J. Exper. Med. **69:** 649, 1939.
(139) Steele, J. M. Proc. Soc. Exper. Biol. and Med. **41:** 86, 1939.
(140) Longcope, W. T. and A. T. McClintock. Arch. Int. Med. **6:** 439, 1910.
(141) Freeman, N. E. and I. H. Page. Am. Heart J. **14:** 405, 1937.
(142) Holman, D. V. and I. H. Page. Am. Heart J. **16:** 321, 1938.
(143) Cannon, W. B. Am. Heart J. **14:** 394, 1937.
(144) Collins, D. A. Am. J. Physiol. **116:** 616, 1936.
(145) Verney, E. B. and M. Vogt. Quart. J. Exper. Physiol. **28:** 253, 1938.
(146) Goldblatt, H., J. Gross and R. F. Hanzal. J. Exper. Med. **65:** 233, 1937.
(147) Goldblatt, H. and W. B. Wartman. J. Exper. Med. **66:** 527, 1937.
(148) Heymans, C., J. J. Bouckaert, L. Elaut, F. Bayless and A. Samaan. Compt. rend. Soc. biol. **126:** 434, 1937.
(149) Alpert, L. K., A. S. Alving and K. S. Grimson. Proc. Soc. Exper. Biol. and Med. **37:** 1, 1937.
(150) Glenn, F., C. G. Child and I. Page. Am. J. Physiol. **122:** 506, 1938.
(151) Glenn, F. and E. P. Lasher. Am. J. Physiol. **124:** 106, 1938.
(152) Bouckaert, J. J., L. Elaut and C. Heymans. J. Physiol. **89:** 3, 1936 (Proc.).
(153) Rogoff, J. M., E. Marcus and P. Wasserman. Proc. Soc. Exper. Biol. and Med. **38:** 199, 1938.
(154) Page, I. H. Am. J. Physiol. **122:** 352, 1938.
(155) Levy, S. E. and A. Blalock. Ann. Surg. **109:** 84, 1939.
(156) Collins, D. A. and E. H. Wood. Am. J. Physiol. **123:** 224, 1938.
(157) Enger, R., F. Linder and H. Sarre. Ztschr. ges. exper. Med. **104:** 10, 1938.
(158) Fasciolo, J. C. Rev. Soc. Argent. Biol. **14:** 25, 1938.
(159) Rogoff, J. M., E. N. Nixon and G. N. Stewart. Proc. Soc. Exper. Biol. and Med. **41:** 57, 1939.
(160) Page, I. H. and J. E. Sweet. Am. J. Physiol. **120:** 238, 1937.
(161) Glenn, F. and E. P. Lasher. Proc. Soc. Exper. Biol. and Med. **38:** 158, 1938.
(162) Tigerstedt, R. and P. G. Bergman. Skand. Arch. Physiol. **8:** 223, 1898.
(163) Collip, J. B. J. Physiol. **66:** 416, 1928.
(164) Pickering, G. W. and M. Prinzmetal. Clin. Sc. **3:** 211, 1938.
(165) Bingel, A. and E. Strauss. Deutsch. Arch. klin. Med. **96:** 476, 1909.
(166) Hartwich, A. and G. Hessel. Zentr. inn. Med. **53:** 612, 1932.

(167) Hessel, G. and H. Maier-Hüser. Verhandl. deutsch. Ges. inn. Med. **46:** 347, 1934.
(168) Landis, E. M., H. Montgomery and D. Sparkman. J. Clin. Investigation **17:** 189, 1938.
(169) Kohlstaedt, K. G., O. M. Helmer and I. H. Page. Proc. Soc. Exper. Biol. and Med. **39:** 214, 1938.
(170) Grossman, E. B. Proc. Soc. Exper. Biol. and Med. **39:** 40, 1938.
(171) Helmer, O. M. and I. H. Page. J. Biol. Chem. **127:** 757, 1939.
(172) Landis, E. M., W. A. Jeffers and E. Shiels. Am. J. Med. Sc. **197:** 739, 1939.
(173) Williams, J. R., Jr., T. R. Harrison and M. F. Mason. Am. J. Med. Sc. **195:** 339, 1938.
(174) Williams, J. R., Jr. and E. B. Grossman. Am. J. Physiol. **123:** 364, 1938.
(175) Williams, J. R., Jr. Am. J. Physiol. **124:** 83, 1938.
(176) Hessel, G. Klin. Wchnschr. **17:** 843, 1938.
(177) Friedman, B., D. I. Abramson and W. Marx. Am. J. Physiol. **124:** 285, 1938.
(178) Boylston, G. A., E. G. McEwen and A. C. Ivy. Proc. Soc. Exper. Biol. and Med. **40:** 559, 1939.
(179) Wakerlin, G. E. and M. Yanowitz. Proc. Soc. Exper. Biol. and Med. **41:** 51, 1939.
(180) Grossman, E. B. and J. R. Williams, Jr. Arch. Int. Med. **62:** 799, 1938.
(181) Friedman, B., B. S. Oppenheimer, E. Somkin, E. T. Oppenheimer and B. Blumenthal. J. Clin. Investigation **18:** 377, 1939 (Proc.).
(182) Williams, J. R., Jr., J. T. Diaz, J. C. Burch and T. R. Harrison. Am. J. Med. Sc., in press.
(183) Merrill, A., J. R. Williams, Jr. and T. R. Harrison. Am. J. Med. Sc. **196:** 18, 1938.
(184) Grollman, A., T. R. Harrison and J. R. Williams, Jr. Personal communication, 1939.
(185) Wakerlin, G. E. and G. R. Chobot. Am. J. Physiol. **126:** P. 646, 1939.
(186) Leiter, L. and L. Eichelberger. J. Clin. Investigation **18:** 477, 1939 (Proc.).
(187) Merrill, A., J. R. Williams, Jr. and T. R. Harrison. Am. J. Med. Sc. **196:** 240, 1938.
(188) Corcoran, A. C. and I. H. Page. Am. J. Physiol. **126:** 354, 1939.
(189) Steele, J. M. and H. A. Schroeder. J. Clin. Investigation **18:** 477, 1939 (Proc.).
(190) Harrison, T. R., A. Blalock, M. F. Mason and J. R. Williams, Jr. Arch. Int. Med. **60:** 1058, 1937.
(191) Prinzmetal, M. and B. Friedman. Proc. Soc. Exper. Biol. and Med. **35:** 122, 1936.
(192) Govaerts, P. and E. Dicker. Compt. rend. Soc. biol. **122:** 809, 1936.
(193) Prinzmetal, M., B. Friedman and N. Rosenthal. Proc. Soc. Exper. Biol. and Med. **34:** 545, 1936.
(194) Page, I. H. Proc. Soc. Exper. Biol. and Med. **35:** 112, 1936.
(195) Collins, D. A. and F. W. Hoffbauer. Proc. Soc. Exper. Biol. and Med. **35:** 539, 1937.

(196) Dicker, E. Compt. rend. Soc. biol. **122:** 476, 1936.
(197) Houssay, B. A. and A. C. Taquini. Rev. Soc. Argent. Biol. **14:** 5, 1938.
(198) Houssay, B. A. and A. C. Taquini. Rev. Soc. Argent. Biol. **14:** 86, 1938.
(199) Fasciolo, J. C., B. A. Houssay and A. C. Taquini. J. Physiol. **94:** 281, 1938.
(200) Mason, M. F. and J. D. Rozzell. Proc. Soc. Exper. Biol. and Med. **42:** 142, 1939.
(201) Wakerlin, G. E. and G. R. Chobot. Proc. Soc. Exper. Biol. and Med. **40:** 331, 1939.
(202) Katz, L. N., M. Mendlowitz and M. Friedman. Proc. Soc. Exper. Biol. and Med. **37:** 722, 1938.
(203) Rodbard, S. Am. J. Physiol. **126:** P. 611, 1939.
(204) Cash, J. R. and J. E. Wood, Jr. South. Med. J. **31:** 270, 1938.
(205) Dill, L. V. and C. C. Erickson. Proc. Soc. Exper. Biol. and Med. **39:** 362, 1938.
(206) Child, C. G. and F. Glenn. Arch. Surg. **36:** 376, 1938.
(207) Levy, S. E. and A. Blalock. Surgery **3:** 899, 1938.
(208) Davis, L. and M. H. Barker. Ann. Surg., In press.
(209) Goldblatt, H. The Harvey Lectures. Series 33, p. 237, 1937–38, Williams & Wilkins, Baltimore.
(210) MacNider, W. deB. and G. L. Donnelly. Proc. Soc. Exper. Biol. and Med. **40:** 271, 1939.
(211) Davis, H. A. and I. F. Tullis, Jr. Proc. Soc. Exper. Biol. and Med. **40:** 161, 1939.
(212) Mansfield, J. S., D. M. Weeks, A. Steiner and J. Victor. Proc. Soc. Exper. Biol. and Med. **40:** 708, 1939.
(213) Abrami, P., M. Iselin and Robert-Wallich. Presse med. **47:** 137, 1939.
(214) Butler, A. M. J. Clin. Investigation **16:** 889, 1937.
(215) Barker, N. W. and W. Walters. Proc. Staff Meet. Mayo Clinic **13:** 118, 1938.
(216) Leadbetter, W. F. and C. E. Burkland. J. Urol. **39:** 611, 1938.
(217) Boyd, C. H. and L. G. Lewis. J. Urol. **39:** 627, 1938.
(218) Barney, J. D. and H. I. Suby. New England J. Med. **220:** 744, 1939.
(219) Karsner, H. T. The New Internat. Clinics **2:** 1, 1938 (Lippincott).
(220) Goormaghtigh, N. and K. S. Grimson. Proc. Soc. Exper. Biol. and Med. **42:** 227, 1939.
(221) Goormaghtigh, N. Bruxells-Medical **19:** 1541, 1939.
(222) Page, I. H. J. Exp. Med. **70:** 521, 1939.
(223) Kohlstaedt, K. G., I. H. Page and O. M. Helmer. Am. Heart J. **18:** 618, 1939.
(224) Harrison, T. R., A. Grollman and J. R. Williams, Jr. Am. J. Physiol. **128:** 716, 1940.
(225) Page, I. H. and O. M. Helmer. J. Exp. Med. **71:** 29, 1940.
(226) Harrison, T. R., A. Grollman and J. R. Williams, Jr. Personal Communication, 1940.
(227) Gerbi, C., B. B. Rubenstein and H. Goldblatt. J. Exp. Med. **71:** 71, 1940.
(228) Mason, M. F., A. Blalock and C. S. Robinson. J. Biol. Chem. (Proc.), xxxiv, lxiii, 1940.

PLASMA PROTEINS: THEIR SOURCE, PRODUCTION AND UTILIZATION

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The subject of this review is one generally shunned by texts on physiology and perhaps this is evidence of wisdom on the part of the authors concerned. Having accepted the responsibility for this review the writers must expose their ignorance, and perhaps that of other workers in this field, and invite critical comment. It will give us both pleasure if this review is helpful to co-workers in this difficult but rapidly expanding field. We express regret if lack of space and time has led to oversight of significant contributions.

It may be stated with confidence that the keen interest in amino acids as relating to protein digestion and utilization in the body actually was responsible for the view that plasma proteins were inert substances having little or nothing to do with tissue nutrition and internal nitrogen metabolism. The evidence now appears convincing that under certain conditions (and probably more or less continuously) there is a "give and take" between body proteins and plasma proteins. For example, a dog while fasting can be maintained in nitrogen equilibrium by plasma protein given intravenously.

The chemical and physical natures of proteins have received much attention in recent years but a review (74) of this knowledge does not yet allow us to describe definitely the proteins under consideration. Although recent evidence from Scandinavian, British and American laboratories (reviewed in (74)) renders it probable that these plasma protein fractions are all part of a single, variably bound plasma protein system, we shall continue to speak of *albumin*, *globulin*, and *fibrinogen*, for they do have a certain *independent importance* in biological reactions. Moreover, to be acceptable new hypotheses of protein structure cannot be incompatible with this independence.

SITE OF FORMATION OF PLASMA PROTEIN. It is our conviction that the liver is of primary importance in the production of plasma proteins but it must be admitted that there is much conflicting evidence in the literature (51, 67, 7).

There is much evidence that *fibrinogen production is wholly dependent upon liver function.* Many experiments with hepatic poisons (chloroform and phosphorus) (18, 22, 64, 88, 29, 75) give irrefutable evidence that when the liver is injured the blood fibrinogen falls rapidly and somewhat in proportion to that liver injury. With liver regeneration and repair the fibrinogen blood values return to normal. Injury to any other tissues (in the absence of liver injury) is followed by *elevation* of the blood fibrinogen. Such experiments are not absolutely conclusive (as are very few observations in physiology) but they cannot be put aside by critics until more convincing experiments pointing to a different interpretation are forthcoming.

Hepatectomized animals give supporting evidence but on the whole no more convincing than the experiments with liver injury. The relatively short life after liver removal and the profound systemic and circulatory disturbances are factors which confuse the picture after hepatectomy (90, 53, 52, 72, 36). The most satisfactory hepatectomy experiments relating to fibrinogen are those of Drury and McMaster (23) and Jones and Smith (36). In dehepatized rabbits (23) a drop in blood fibrinogen was recorded—35 to 65 per cent below normal in 15 to 30 hours. In dogs (36) there was a definite and progressive fall in fibrinogen to 16 to 47 per cent below the control levels within 13 to 20 hours after hepatectomy. It must be admitted that we do not know how rapidly fibrinogen is used in the normal and particularly in the abnormal animals and this lack of knowledge weakens the deductions drawn from the hepatectomy experiments.

One who is acquainted with the literature hesitates to bring up the subject of the *origin of albumin and globulin.* In the case of fibrinogen the opinions center more or less about the liver but in the debates about the source of albumin and globulin hardly an organ or tissue escapes, unless it be the brain. And in fact it may be that under certain circumstances almost any tissue can contribute to a globulin fraction. We prefer to state our own convictions first and submit arguments relative to these beliefs before mentioning some of the other views. The present evidence speaks in favor of the liver as the site of production of albumin and of much of the globulin. It must be admitted that many proteins appearing in the globulin mixture may be derived from body cells other than the liver.

The evidence pointing to the liver as the site of albumin and globulin formation is both clinical and experimental. Some years ago Kerr, Hurwitz, and Whipple (39) noted a definite lag in the regeneration of

serum protein of Eck fistula dogs following acute plasma depletion. More recently Knutti, Erickson, Madden, Rekers and Whipple (40) reported observations over two years on an Eck fistula dog. Always in excellent clinical condition, this dog at times was unable to form new plasma protein in significant amounts on various standard diets; in fact, on occasions this dog could form only about one-tenth as much plasma protein as normal controls. At autopsy, the only significant findings were a perfect Eck fistula and the usual atrophic liver found in these dogs.

Hepatectomy experiments (15, 27) have added little significant evidence relating to plasma albumin and globulin. Partial hepatectomy in rats (17) is followed by depression of plasma protein within 24 hours. It would be interesting to know something about blood volume at this time in these rats. The albumin fraction remains low for the subsequent 4 weeks and this observation may be significant. Moreover, Warner, Brinkhous and Smith have found that either partial hepatectomy or chloroform injury results in a marked decrease in the plasma prothrombin, an important one of the many globulins (81, 80).

Clinical observations point to the liver as the source of plasma proteins (14). A child with edema and *hypoproteinemia* was observed for 7 months and at autopsy Thompson, McQuarrie and Bell (77) observed liver atrophy with disappearance of cells in intermediate and peripheral zones in the lobules. The other tissues were normal. Johansen (35) added a case of idiopathic hypoproteinemia which at autopsy showed an *interstitial hepatitis.* Cases of liver cirrhosis often show a low level of plasma protein, especially a low albumin:globulin ratio.

One almost perfect experiment is frequently overlooked by physiologists who regard the bone marrow as the source of plasma proteins. Human cases of *aplastic anemia* do come to autopsy with almost complete absence of all marrow elements yet with *plasma proteins all* within normal range. This would seem to dispose of the marrow cells (7) as an essential factor in plasma protein fabrication.

Hyperproteinemia has been recorded in a variety of clinical diseases—myeloma (12), osteomyelitis, lymphogranuloma inguinale (89), syphilis. The globulin fraction is usually responsible for this increase and evidence (12) has been submitted to show the presence of an abnormal globulin. Qualitative changes in the serum proteins in renal disease (nephrosis) have been found by immunological (30) and precipitation experiments (34).

Perfusion experiments are difficult to evaluate (11, 60) but may pre-

sent intriguing data (19). Cutting and Cutter (19) found that injection of india ink abolished the ability of the rat to replace acute loss of plasma protein. We do not regard the reticulo-endothelial system as major factor in plasma protein formation but a growing body of evidence, such as that offered by Sabin (73), indicates that some globulin can be made in body cells outside of the liver. Arguments pro and con for a transformation of albumin into globulin or vice versa cannot be reviewed at this time (61, 28, 82, 24, 7).

MATERIALS CONCERNED IN THE FORMATION OF PLASMA PROTEINS. It seems obvious that protein or its derivatives, including amino acids, must be furnished the animal organism to achieve the production of new plasma protein. Certain protein already within the body as well as some of that received orally (or parenterally) may be commandeered for plasma protein formation. The exogenous supply will be given first consideration.

Food protein influences plasma protein production. Clinical observations substantiating this statement are numerous and have been reviewed by Moschcowitz (63). The hypoproteinemia and edema of malnutrition are cured by the ingestion of adequate food, provided that there are not also present any of the adverse factors—infection, liver disease (see below). Kerr, Hurwitz and Whipple (38) offered the first experimental evidence that diet influenced favorably the regeneration of depleted plasma proteins. In dogs *adequate* food protein has more recently been found to have a *qualitative* as well as a quantitative significance (65, 85). Per unit of protein fed, beef serum will favor the production of 3 times as much plasma protein as beef heart and more than 5 times as much as beef stomach. Let us examine the evidence upon which this statement is based.

If one places a dog upon a constant diet adequate in calories and other essentials but limited in protein to about 1 gram per kilo per day, the animal may gradually be depleted of plasma protein by daily plasmapheresis. If at first the dog is bled daily about one-fourth of its blood volume with reinjection of sufficient plasma-free red blood corpuscles suspended in a physiologic salt solution (plasmapheresis) to prevent anemia, a plasma protein concentration of 4 grams per 100 cc. will soon be reached. If the subsequent daily plasmaphereses are so adjusted as to maintain steadily this low plasma concentration, it will be found that smaller and smaller bleedings will suffice, but that a minimum will shortly be reached. This minimum, expressed as the grams of *plasma protein* removed from the dog during the course of one week,

may be called the *basal output.* This minimum quantity will remain constant for a given dog on a given basal diet for as long as the experiment lasts, and this period can cover at least a year (49).

If variations are introduced into the existence of this dog, a standardized biologic machine, their effect can be measured in a change in the quantity of plasma protein which must be removed from the animal in order to maintain standard conditions. Thus, if beef serum is added to the basal diet, a quantity of plasma protein will be produced in addition to the minimum basal output, equal to about 40 per cent of the protein in the beef serum fed (65). Such a response leads one to believe that the supra-basal, new-formed plasma protein comes (in whole or large part) from materials furnished in the added diet protein, and that the *basal diet protein* is wholly responsible for the *basal* output of plasma protein. In addition, variations in the quantities of plasma protein obtained from the feeding of different proteins justifies the assumption of qualitative differences.

Table 1 summarizes the proteins which have been tested in the formation of plasma protein. The tests represent the work of three different laboratories and as many different methods. The larger group of tests has been done in this laboratory by the method described above and the results are expressed as *grams of new plasma protein resulting from the feeding of 100 grams of the test protein.* When the test protein was the only protein in the diet, a "‡" follows the potency value; otherwise all test foods were added to a basal adequate protein intake. Thus, "liver, pork, raw, 17–33‡" indicates that when raw pork liver constituted the total protein of basal diets tested, the per cent return of plasma protein ranged from 17 to 33, in different dogs, not in the same dog. Reasons for such differences will be discussed below. Other differences will be noted. In one dog eating a basal ration containing the proteins of potato and bran flakes, added kidney protein was only one-fourth as well utilized for plasma protein formation as in another dog consuming a basal diet containing kidney. Conspicuous differences of this type are unusual and difficult of interpretation.

That plasma protein formation can be controlled by diet and that different proteins have different values in plasma formation are verified by an entirely different method (Weech and Goettsch, 84, 85, 83). These workers found that on a diet containing inadequate protein, furnished by carrots and polished rice, a decline in the serum albumin concentration occurred which was parallel to the decline in total serum protein concentration and also parallel to the plasma volume. If then

at the end of 3 weeks' decline a test protein were added to the diet, its effect on the serum albumin concentration could be measured during the fourth week. When this effect was determined for the same food on a number of different dogs, a rather wide range of biological response was found, but significant averages were established. For example, the

TABLE 1

*The potencies of various food proteins in forming blood plasma protein. Summary of all published data**

REFERENCE	HIGH POTENCY	MEDIUM POTENCY	LOW POTENCY
31, 65, 55, 47, 48, 49, 50, 91 (Rochester)	Serum, beef, 38† Serum, beef, dried, 28+	Salmon bread, 24††‡ Yeast, fresh, autoclaved, 23 Bran Flakes, 23 Kidney, pork, cooked, 22 Liver, pork, raw, 17-33‡ Kidney, pork, cooked, 19‡ Rice polishings, 19 Gizzard, 19 Thyroid, powdered, 19 Rice, polished, 19‡ Lactalbumin, 18 Skeletal muscle, beef, 18 Egg white, 17 Irish potato, powdered, 16 Salmon, 16‡ Liver, pork, raw or cooked, 15 Soy bean, 14, 8 Heart, beef, 13 Casein, 12 Liver extract, 12	Spleen, 10† Red blood cells, dog, 10 Brain, pork, 8 Stomach, beef, 7 Salmon, canned, 7 Gelatin, 2, 9 Pancreas, 6 Kidney, pork, cooked, 6 Zein, 0
83 (Columbia)	Serum, beef, 0.801	Egg white, 0.613 Beef chuck, 0.475 Beef liver, 0.436 Casein, 0.388	Gelatin, −0.093
59 (Yale)	Serum protein, beef, 0.53 Casein, 0.45 Lactalbumin, 0.38		

* See text for discussion.

† Figures mean grams of new plasma protein resulting from feeding of 100 grams test protein.

‡ Signifies potency as *basal* protein; all other figures indicate tests as *supplementary* protein.

assay values for beef serum in 11 different dogs ranged from 0.42 to 1.27, with an average value of 0.801; whereas the assay values for casein tested in 12 different dogs ranged from 0.07 to 0.58, with an average ("potency value") of 0.388, a statistically valid difference as pointed out by Weech. The potency values for other foods tested by this method are given in table 1 (Columbia). The biological variation

displayed by different animals under this test is of considerable interest. It may account for some of the sharp variations in assay values listed in table 1 from the writers' laboratory.

These potency values of Weech measure relative differences in *rate* of formation and not in total capacity for formation of albumin, as is measured for plasma proteins by the plasmapheresis technique. Evidently in the materials so far tested these two qualities are proportional, for the relative orders of potency determined by the two methods are similar. Such may not be true in all instances. It has been frequently noted that the addition to the basal diet of some proteins, for example soy bean, is followed by a much *prompter response in production of new plasma protein* than the addition of other equal or even superior materials, such as "gelatin + cystine + tyrosine" (table 2). The published data giving the *weekly totals* (55, 49) tend to obscure, though not entirely, this phenomenon so clearly observed in the daily determinations.

Melnick, Cowgill and Burack (59) assayed serum protein, casein, and lactalbumin and concluded that there was no significant difference in any of their potency values (see table 1). Their test procedure employed plasmapheresis and a basal protein-free diet (58). During the first week of this regime, removing daily 25 per cent of the blood volume of the dog, the circulating concentration of serum protein is reduced to 4 per cent or less. During the subsequent week of protein-free diet serum protein is removed as necessary to maintain the concentration near 4 per cent. In the third week, the test protein is added to the protein-free diet and in the fourth week the relation of serum protein removed to test protein intake is compared with the figures obtained for the second week and a potency ratio is calculated. For validity, their method rests on two assumptions. It is assumed first that of the total quantity of test protein fed, the fraction used for body nitrogen requirements other than serum protein formation can be determined, and this quantity is thought to be the minimum amount of the test protein which will keep the same dog in nitrogen equilibrium when it is in a normal non-hypoproteinemic state (57). Secondly, the reserve store of plasma protein building material of any dog is assumed to be measured as that quantity of plasma protein which can be removed in 6 bleedings during one week, each bleeding being equal to one-fourth the blood volume. As an important factor contributing to the formation of plasma proteins, the *reserve store* is discussed below. We believe the reserve store of plasma protein building materials cannot be removed

completely by this rapid procedure and that the assays recorded (59) are inaccurate because of the presence within the body of an unknown amount of protein reserve stores clearly evident in later experiments (56).

Do some foods favor albumin production? In 1932, Liu and co-workers (43) found animal protein twice as effective as vegetable protein in combatting edema and hypoproteinemia, but in a later publication, Liu and Chu (42) could find only slightly more favorable action in animal protein than in vegetable protein in raising the plasma protein concentration of two patients with nephrosis. As a matter of fact, neither type of protein was very definitely effective in this regard although a similar and considerable nitrogen retention and gain in body weight was obtained by each. The gradual rise in plasma protein concentration which occurred in both cases is probably better attributed to removal of incident infection than to dietary measures.

Animal experiments testing the relative capacities of animal and plant proteins in albumin and globulin formation present conflicting evidence (85, 55, 47). In long continued plasmapheresis experiments by which hypoproteinemia is maintained in dogs, the albumin:globulin ratio is almost invariably below normal. This reduction in the albumin:globulin ratio is found regardless of the kind of protein fed, although it may be greater with certain plant or grain proteins, ranging from 0.3 to 1.2. Only five different protein-containing foods of plant origin have been tested in such experiments (see table 1). These are soy bean, potato, bran, rice polishings, and rice. Soy bean is in a class by itself and is very promptly utilized and favors a high albumin:globulin ratio like many animal proteins. Only soy bean and rice have been fed unaccompanied by other protein and sample albumin:globulin ratios for the plasma protein produced are 1.14 for soy bean contrasted to 0.51 for rice (55). The soy bean was fed at an intake level of 1.9 grams protein per kilo body weight in a different dog. Such differences in intake may be of no significance as it has been observed that the albumin:globulin ratio during liver feeding is not appreciably altered by tripling the intake (dog 32-130 (65) compared with dog 34-152 (55)). Four dogs (65, 55) receiving protein from a potato-bran diet mixture had albumin:globulin ratios in basal periods ranging from 0.47 to 0.88. For comparison 4 other dogs (55, 47, 48, 49) receiving protein only from kidney or liver had basal albumin:globulin ratios ranging from 0.58 to 1.10. The lengths of these experiments and the intake levels of protein were comparable. It is evident that for albumin production,

sweeping conclusions concerning the group superiority of animal or of plant proteins cannot be safely drawn. However, certain plant proteins (rice and potato) in these experiments do favor a low albumin:globulin ratio or appear to favor the production of globulin when compared with certain standard animal proteins (meat, kidney and liver).

When a fasting dog is given plasma protein (whole dog plasma) by vein, the dog is kept in nitrogen equilibrium and even in weight equilibrium for many days (21). The dog uses up the introduced protein to supply its protein needs and as this goes on day by day the *albumin: globulin ratio remains unchanged* (66). This indicates that the body can and does use both albumin and globulin at about the same rate to carry on its normal internal protein metabolism. This would suggest that differences in the albumin:globulin ratio might be more frequently due to *variations of production* rather than to lack of use of the normal globulins.

Weech and Goettsch (84) find that in their method of assay, described above, the albumin and not the globulin fraction of the serum is influenced by the character of the diet. They do find an increase in the quantity of circulating globulin during the feeding of test proteins, but conclude that diet is not an important factor in influencing the formation of globulin.

Under the experimental circumstance of plasmapheresis, there are data which indicate that globulin formation is directly dependent on the diet (31, 65). For example, the same 100 grams of beef serum which produced 38 grams total plasma protein, produced approximately 21 grams albumin and 17 grams globulin (65). The addition of 100 grams bran flakes to a kidney basal diet (55) results in the formation of about 12 grams albumin and 11 grams globulin. Since it has been shown that 100 grams casein will yield only 5 grams albumin and 7 grams globulin, and 100 grams gelatin 5 grams or less of each, it becomes apparent that as measured by plasmapheresis *diet regulates globulin production equally as well as albumin formation.*

Amino acids. Since the experiments of O. Loewi in 1902 (44), demonstrating that the feeding of protein digests could maintain nitrogen equilibrium, much effort has been directed toward discovering the dietary essential protein constituents. For the growth of rats, these essential amino acids have been recently determined in the laboratory of W. C. Rose in a series of experiments announced by him in 1937 (69, 70). Rats fed a diet, the nitrogen of which is furnished by a mixture of pure amino acids, exhibit normal growth curves only when

appropriate quantities of the following ten amino acids are included in the mixture: threonine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophane, arginine, histidine, and lysine. Moreover, Rose states that normal growth occurs if these amino acids are offered to the exclusion of all others. Such experiments invite speculation regarding the amino acid requirements for plasma protein synthesis. Since these ten amino acids support growth, they probably support the formation of new plasma protein in the growing blood volume of the young rat. Direct evidence of such a synthesis would be valuable.

In the dog by the plasmapheresis technique described above direct measurement of new formed protein can be made. Certain amino acids have been studied for their effect on the formation of new plasma protein in such standardized dogs (47, 48, 49, 50). When the addition of one or more amino acids to a basal diet is followed by an increase in plasma protein formation above basal it may be inferred that the added amino acids supplement the mélange available to the synthesizing mechanism (liver probably) from diet and body sources in such fashion as to permit the formation of more new protein. It seems probable that the added amino acids are at least in part incorporated in the new plasma protein and thus represent components essential for its synthesis in a depleted dog put to the strain of maximal regeneration of plasma protein. Whereas, positive experiments of this type are significant, it is obvious that a negative response does not imply that the added amino acid is unessential for plasma protein synthesis.

In table 2 are recorded the results of such amino acid feeding experiments. a, *Cystine*, with tryptophane or tyrosine, adds much plasma protein producing power to gelatin; b, *cystine*, with tryptophane and the other amino acids indicated, adds much plasma protein producing power to zein; c, *cystine*, with glycine, glutamic acid, and leucine adds much potency to the liver basal diet. Under certain conditions cystine qualifies as a *key amino acid* in plasma protein regeneration.

The last observation (c) raises a question as to the assumed participation of gelatin and zein in the first (a) and second (b) reactions. Calculated on the same basis (see footnote *, table 2), the potent amino acids with gelatin increase the basal output 121 to 191 per cent, with zein 108 and 134 per cent, and without either 107 to 121 per cent. Upon examining the original protocols (49, 50), however, the urinary nitrogen figures indicate a conservation of gelatin nitrogen of 40 to 70 per cent and of zein nitrogen of 90 per cent (of the added amino acid nitrogen of 100 per cent). It appears, therefore, that in these experiments the

TABLE 2

Summary of experiments on the influence of certain amino acids in plasma protein formation

	EFFICIENCY IN PLASMA PROTEIN FORMATION: PROTEIN OUTPUT PER CENT OF PROTEIN INTAKE			
Reference	(49, 50)	(49)	(48)	(47)
Dog number	36-196	37-6	33-11	34-152
Basal diets				
Liver	17	27	22	
Kidney				22
Supplements				
Gelatin + cystine + tyrosine + tryptophane	34			
Gelatin + cystine + tyrosine	39, 25			
Gelatin + cystine + tryptophane	32	41		
Gelatin + cystine	10			
Gelatin + tyrosine	5			
Gelatin + tryptophane	3±	3	33	
Gelatin + tyrosine + tryptophane	1			
Gelatin + cystine + phenylalanine	6	13		
Gelatin + methionine + tyrosine	12, 21			
Gelatin + methionine	5			
Zein + cystine + tryptophane + lysine + glycine + threonine	22			
Zein + cystine + tryptophane + lysine + glycine	28			
Tryptophane	0*		0*	
Lysine				0*
Histidine + lysine + arginine				26*
Cystine + glycine + glutamic acid				50*
Cystine + glycine + glutamic acid + leucine	107*			
Cystine + glycine + glutamic acid + leucine + isoleucine + arginine + lysine	117*			
Cystine + glycine + glutamic acid + leucine + tyrosine	121*			

* These figures represent the percentage increase in the basal plasma protein output induced by the amino acid supplement.

gelatin and the zein do participate effectively in the protein metabolism. It seems that the content of cystine, and probably leucine in liver limits the potency of the liver basal ration. Methionine deserves further

testing. Present indications are that methionine is not an efficient substitute for cystine in plasma protein formation, nor is phenylalanine for tyrosine. These experiments do not prove methionine or phenylalanine to be dispensable in plasma protein formation nor by their apparently negative reactions is such indicated for lysine, isoleucine, arginine, or threonine. In a preliminary report Rose and Rice (71) announce that nine amino acids are essential for maintenance of weight and nitrogen balance in the adult dog, the same nine amino acids which together with arginine Rose and co-workers found essential for normal growth in the rat (69, 70).

It appears reasonable to expect that in the near future a method will be perfected for the *parenteral administration* of the nitrogen requirements of the body including that for plasma protein formation. Its practical value in treatment of certain disease states is obvious. Observations by Elman (25) on dogs indicate that serum protein regeneration following severe hemorrhage may be aided by the intravenous administration of hydrolyzed casein with added tryptophane. Elman and Weiner (26) have carried out similar intravenous infusions on patients suffering from malnutrition and hypoproteinemia. They report that edema usually disappeared, serum protein concentration occurred, and nitrogen balance was easily achieved as a consequence of the casein digest injections. They found that these favorable results were not obtained when tryptophane and cystine (or methionine) were not added to the digest.

Miscellaneous exogenous materials tested in plasma protein regeneration. As would be expected in the normal animal, no substance has been found which will substitute for protein or amino acid in plasma protein synthesis. A recent report (68) indicating some utilization of *ammonia nitrogen* in body protein synthesis should prompt an early trial of such substances in plasma regeneration. Certain α keto and α hydroxy acids corresponding in other respects to essential amino acids have been found to substitute satisfactorily for such amino acids in the synthesis of body protein (70) and presumably the appropriate ones could be used in plasma protein formation. Parenteral *liver extract* and iron have both proved ineffective in stimulating regeneration in hypoproteinemic dogs (47).

ENDOGENOUS MATERIALS CONCERNED IN THE FORMATION OF PLASMA PROTEINS. *A reserve store of plasma protein building material is one subject for which the evidence is overwhelming and it may be accepted as a fact.* That such materials exist was first demonstrated by Morawitz

(62) in 1906, when he found that regeneration of the blood plasma proteins occurred during fasting following their acute depletion by bleeding. He combatted anemia by injection, simultaneous with the bleeding, of an equal quantity of red blood cells suspended in Locke's solution to which 3 per cent gum acacia had been added. This was the first use of a technique, later called *plasmaphaeresis* by Abel, Rowntree and Turner (1), which has proved valuable in studying the blood proteins. Morawitz found that by a large one-stage plasmapheresis the total plasma proteins of a dog could be reduced to about 2 per cent and that return to a normal level occurred during fasting, in 2 days in one instance and in 4 days or less in another. In the second instance, the dog was depleted again at 4 days, this time with reduction of the total plasma protein concentration to 3 per cent, and regeneration was observed, reaching a level of 5.4 per cent in 3 days, all in the fasting animal.

Kerr, Hurwitz and Whipple (37) and Smith, Belt and Whipple (76) added further evidence of a similar type to indicate that after rapid experimental depletion plasma protein regeneration can occur in the fasting dog. From such experiments it can be calculated that the quantity of materials available is at least sufficient to reform 40 to 60 per cent of the circulating mass of plasma protein originally present. By a similar approach but in a different species (rat), Cutting and Cutter (19) found that 40 per cent of the original mass of circulating protein could be regenerated within 12 hours after its removal. Seven days' fasting did not alter this remarkable response.

By another method in this laboratory, measurement of the *reserve store* of plasma protein building material has been made in many dogs (31, 65, 55, 47). The method and the calculation determining this store have been described in detail (48). The normal dog is depleted of circulating plasma protein by daily plasmapheresis while consuming a constant basal diet low but adequate in protein. With the normal dog it will be found necessary to remove more plasma (and plasma protein) in the initial days or weeks of the regime than in subsequent weeks in order to attain and keep a steady hypoproteinemia. This *excess quantity* of plasma protein removed in the first weeks of a prolonged period of plasmapheresis represents the *reserve store.* It is a greater quantity of protein than is represented by the difference in circulating mass of plasma protein before and during the experiment (i.e., (6 per cent − 4 per cent) × plasma volume = about 9 grams plasma protein in a 10 kgm. dog). Its measurement is graphically represented in figure A. The excess production noted in the initial 4 weeks (fig. A) must be

attributable to protein materials present in the body at the start of the experiment.

Probably largely influenced by the preceding nutritional history the quantity of *storage materials* available for plasma protein production has been found to vary rather widely from dog to dog. In actual experiments, the maximum quantities of reserve protein stores have usually not been measured since in the endeavor to attain basal conditions as rapidly as possible the animals have been *fasted* during the initial week or have been depleted by a previous period on a low protein diet.

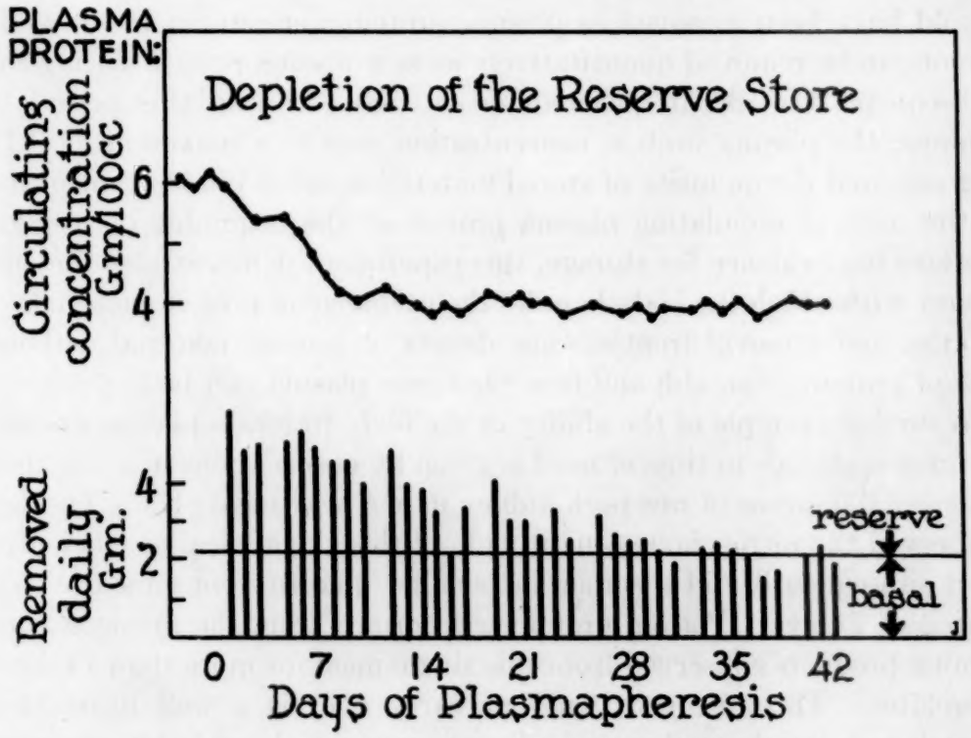

FIG. A

Thus the figures actually obtained are low. In 10 dogs, 10 to 14 kgm. in weight, the plasma protein production from reserve stores ranged from 24 to 68 grams (92). On other occasions in 2 of these dogs after both low protein diet and fasting, the reserves amounted to only 9 and 11 grams. Two larger dogs of 16 and 21 kgm. had reserves of 121 and 108 grams (30). It may be estimated that normal dogs have sufficient materials in storage to form a quantity of plasma protein one to two times that normally present in their circulations. This may amount to as much or more than the total protein content of the liver and indi-

cates that a large portion of it must be stored elsewhere. This amount is larger than that given by plasma protein regeneration during fasting (62, 37, 76) and probably more nearly represents what the animal can produce when the *only demand* on the stores is for plasma regeneration. Dogs once subjected to plasma depletion and thereafter allowed to return to normal exhibit *larger reserve stores* on subsequent depletion (48).

Other experiments reveal the process of *storage of plasma protein* forming materials. Such stores can be experimentally induced and reduced quantitatively. In a standardized protein depleted dog, plasmapheresis was discontinued for 2 weeks and the materials which ordinarily would have been removed as plasma protein were stored in the body depots, to be removed quantitatively as new plasma protein during the subsequent 3 weeks of plasmapheresis (48). During this period of storage, the plasma protein concentration rose to a maximum of 4.75 per cent and the quantity of stored material equaled 150 per cent of the entire mass of circulating plasma protein at the beginning of storage. Besides the evidence for storage, this experiment demonstrates the efficiency with which the body handles the exchange of protein materials—storage and removal from storage depots of protein material without loss of protein—"an ebb and flow" between plasma and body proteins.

A further example of the ability of the body to retain plasma protein forming materials in time of need is given by observations in a dog that ate over 400 grams of raw pork kidney in one brief meal (47). The dog conserved the nitrogenous constituents of this single meal as efficiently as it subsequently did a somewhat smaller quantity consumed over a period of 7 days. Plasma protein was formed from the nitrogen containing products conserved from this single meal for more than 14 days thereafter. This nitrogen "lag" or carry over is a well recognized though not clearly understood phenomenon which probably includes protein storage and the "ebb and flow" of proteins in the body as the terms are used in this review.

We have considered in the preceding paragraphs data bearing on the question of a body store of materials for *plasma protein synthesis*. Since these stores may be lessened by low protein diets and fasting, it is reasonable to suppose that they are a part of the general body stores measured by Addis, Poo and Lew (3, 5). The whole subject of protein storage has been reviewed by Cathcart (16) to 1921 and more recently by Luck (46).

Nature of the protein reserve store. A complete discussion of the nature of stored protein is beyond the scope of this paper. That the ni-

trogenous metabolites are stored as protein appears probable from the weight of evidence reviewed by Borsook and Keighley (13). These investigators present new data to indicate that in an adult man in nitrogen equilibrium and a urinary nitrogen excretion about 10 grams daily about half of this nitrogen comes from catabolism of stored protein, the extent of which storage is a function of the previous dietary history. This fraction of the total they term the "continuing" nitrogen metabolism. In this dynamic picture, extensive *synthetic processes* involving amino acids balance the *catabolic portion* of the "continuing" nitrogen metabolism. While the data are meager, the hypothesis is stimulating.

In the utilization of the reserve store for general nitrogen requirements or for plasma protein formation, it is characteristic that a large bulk of the store is used early and rapidly, a so-called "labile" portion. Three illustrations may be cited. 1. Addis, Poo and Lew (4) found that half of the total protein of the liver to be lost during a 7 day fast, is lost during the first 2 days. Considering that 40 per cent of the protein content of the liver of a rat is lost during a 7 day fast (5) liver storage protein appears readily available. Its rate of conversion is probably no greater, however, than that of the greater mass of stored protein found in the carcass of the rat. This mass lost 4 times as much protein as did the liver and from the data given it was available at the same rate, 50 per cent within the first 2 days. 2. Following rapid severe depletion of the plasma proteins in experiments discussed above (62, 76) the rate of regeneration from stored protein was most rapid in the first hours. 3. In the depletion of the reserve stores (48) the larger proportion is removed during the initial week or two but smaller quantities may continue to be contributed by them for as long as 5 or 6 weeks.

An explanation for the above phenomenon may involve the law of mass action and a tentative proposal may be advanced. When the steady state existing between body protein supply and demand is disturbed the rate of the reaction in the direction of a new equilibrium is determined by the product of the active masses of the substances reacting as well as by other constants and conditions (54). Thus, when the mass of stored protein is large, and change of diet, plasma depletion, body injury, or other disturbing factor is introduced, this mass will be converted at a faster rate than when the mass diminishes, other things being equal. Under such a concept the "labile" portion is merely that portion of the reserve store which is mobilized first and fastest and in other respects is not different from that made available more slowly.

Observations on the rate of mobilization of protein stores in response to acute demand for plasma protein indicate that the greater bulk of this reserve is not stored as plasma protein nor as material more easily converted into plasma protein than orally ingested protein. A small portion of the store sufficient to raise the concentration 0.5 per cent within 15 minutes after rapid acute plasma depletion (76) may be essentially preformed plasma protein. If a large portion of the store were readily available in only slightly modified form one would expect a rapid appearance of the plasma protein in the blood stream and a more prompt return to normal plasma protein concentration. Actually dietary protein (38) or intravenous amino acid mixtures (25) will accelerate the relatively slow regeneration of plasma protein from reserve stores.

A review of experiments measuring the reserve store of plasma protein forming materials (92) indicates that the store yields *albumin* on depletion in slightly more abundance than globulin, perhaps 10 per cent greater. This gives us a little insight into the potentialities of the reserve store.

Many investigators believe that to justify the term the *stored protein* should exist as a physically demonstrable entity comparable to glycogen and fat intracellular masses, and some claim to have found such protein deposits (8, 41). The careful chemical analysis of Luck (45) of the protein of the rat liver after storage had been induced, indicates all fractions of this protein to have participated equally in the storage process. Until more is known of the nature of protein as it exists within the cell, further discussion does not seem relevant.

Can plasma proteins be formed from essential (non-storage) body protein? The evidence so far speaks against this possibility, as it has been found that only 3 to 4 grams' plasma protein can be removed from a protein depleted dog during a week of fasting. This limited production is somewhat surprising as it is readily demonstrated that in experimental anemia a standard dog can produce large amounts (40 to 50 grams) of hemoglobin during fasting periods (20). It is probable that these anemic dogs do have some reserves of stored protein but the reaction is conspicuous and uniform and appears to involve conservation of protein-split products which contribute to the nitrogen fraction in the urine of the control dog (20).

The reserve store of protein may be defined as all protein which may be given up by an organ or tissue under uniform conditions without interfering with organ or body function. This definition indicates primary physiological significance, not anatomical. In view of the above

concept we may consider pertinent data. As noted above, dogs may be kept hypoproteinemic by plasmapheresis for months at a time and remain apparently normal in all other respects. *Such dogs are stripped of their protein stores and during periods of fasting or protein-free diet feeding can produce but little plasma protein.* What little plasma protein is removed may be attributed in part to a carry over from the preceding basal diet feeding or to plasma volume concentration or to uncontrollable variation in basal conditions. The quantity removed in one week during fasting ranges from 3 to 6 grams (65, 55) and during protein-free diet weekly periods averages 3 grams (48, 49). Even with the addition of the catabolism of an experimental *abscess* to a fasting, storage-depleted dog, only 4 grams plasma protein were removed in a carefully controlled experiment (50) corresponding to one previously reported (48) in an inadequately depleted dog. The presence of the abscess may introduce as a confusing factor an inhibition of the protein-forming mechanism. The abscess also makes available large quantities of protein split products, but apparently these are not used to form new plasma protein.

An observation by no means conclusive but of some interest in this consideration is one involving the catabolism of hemoglobin. When laked red blood cells were injected into the blood stream of a standard protein depleted dog, there resulted no production of new plasma protein which could be attributed to the injected material (49). This is an example of the failure of catabolism of an essential body protein to supply materials for plasma protein synthesis. Hemoglobin is admittedly an incomplete protein, among other deficiencies being low in cystine, and the lack of this amino acid may actually explain this reaction.

All in all we can give no adequate explanation why the plasma protein depleted dog cannot use effectively some of the waste products coming from protein wear and tear or disintegration. The dog is badly in need of new plasma protein and presumably the stimulus is maximal but the net output of this protein approximates zero when the food protein intake is zero.

MECHANISM OF PLASMA PROTEIN FORMATION. The path leading to the formation of plasma proteins is obscure from the moment the amino acids enter the cell. *Amino acids* liberated by protein digestion are the ultimate source of the essential nitrogen and the preponderance of present opinion holds them to be the simplest units in the syntheses, although Alcock in a thought-provoking argument (6) questions this

assumption. Presumably given the proper mixture of amino acids the normal liver can form and release into the blood stream albumin, globulin and fibrinogen molecules. Bergmann (9) has contributed to our knowledge of the enzymatic processes which may be involved in this synthesis and further developments are eagerly awaited.

It is a truism that cells must form or synthesize protein if the body is to live. Any disturbance of this *mechanism* of protein formation is of great interest (10, 86) but conclusive evidence is hard to find. The fact that there can be an "ebb and flow" between plasma and cell proteins makes it difficult to produce evidence which is "leak proof."

Clinical evidence of long continued hypoproteinemia (77, 35) with demonstrable liver abnormality probably indicates an impaired protein forming mechanism. The Eck fistula liver with limited protein production under standard conditions (40) gives similar evidence favoring an impaired protein formation. There is also some experimental evidence that *infection* can limit the production of plasma protein in well standardized plasma depleted dogs (47). Presumably this means that the plasma protein forming mechanism is disturbed by the complicated body reaction to infection.

Prolonged hypoproteinemia per se causes no damage to the protein forming mechanism (56, 49). The lowered production of plasma protein noted in one report (47) was probably due to some deficiency in the diet not yet understood. Nor does any injury accompany the removal of plasma protein reserve stores by plasmapheresis. On the contrary, three different dogs subjected to long plasma depletion followed by a long rest period on kennel diet, subsequently in the next year when again subjected to plasma depletion showed much *larger reserve stores* of plasma protein building materials (compiled from 55, 47, 48, 49, 50). Moreover, the concentration of plasma protein has been found higher after recovery from long continued depletion than prior to it.

THE DISTRIBUTION OF BODY PROTEIN—A DYNAMIC EQUILIBRIUM. In evaluating the clinical and experimental data relating to plasma protein formation no knowledge would be more useful than an understanding of the processes involved in the distribution of protein within the body. The prevailing attitude appears to be that concentration of plasma protein is a direct measure of production and utilization. The tacit assumption has been that once formed unless obviously lost by hemorrhage, proteinuria, transudation, or inflammation, *plasma protein is static*, having only physico-chemical and immunological functions. We believe that recent evidence demonstrates plasma protein to have

another important rôle. It is a part of a *balanced system of body proteins.* A *steady state* or "ebb and flow" exists between it and a portion of the cell and tissue body protein. This portion called the *reserve store* has been described above. Since no better term has appeared than the one originally given to this steady state (32) we shall refer to it here as a *dynamic equilibrium.*

The evidence for this concept rests upon experiments which upset this dynamic equilibrium. We have noted above the circumstances under which protein materials may flow from cells and tissues into plasma. Experiments accelerating the flow from plasma into tissue are equally informative. By injection of dog plasma into protein-starved dogs, nitrogen equilibrium has been maintained for the periods of the experiments, 14 to 20 days (32, 66, 21), and probably could be maintained indefinitely. Obviously, such parenterally introduced protein is metabolized. Addis (2) has found it to increase the total protein content of the body. Of great interest are the observations by Howland and Hawkins (33) which demonstrate that the metabolism of the injected plasma protein does not result in an increase in blood amino acid concentration sufficient to be detected as increased urinary nitrogen and dextrose (phlorhizinized dogs). The control oral administration of plasma to a phlorhizinized dog was followed by the expected prompt and considerable increase in urinary nitrogen and sugar.

How are we to interpret such experiments? The injected plasma protein disappears from the circulation within 24 hours or less. The urinary nitrogen and sugar figures for 48 hours after injection of plasma compare with those obtained in the same dogs following injection of Locke's solution. It appears that the *metabolism of intracellularly ingested plasma protein* does not increase the flow of amino acids into the blood stream. Therefore, it appears probable that it does not increase the amino acid concentration of the tissues. But it can supply all of the nitrogen requirements of the body.

To return to the plasma-injected non-phlorizinized dogs, it is obvious that in nitrogen equilibrium they excrete more urea and ammonia nitrogen during injection periods than during periods of protein starvation. This increased nitrogen excretion must come either from catabolism of intracellularly ingested plasma protein or from increased catabolism of cell protein attendant upon the conversion of plasma protein to cell protein, or from both.

Speculation may be permitted as to the metabolic procedure of conversion of plasma protein to cell protein. It would appear simpler for

the body to split only partially and then realign the *large aggregates* into cell protein rather than be forced to digest such protein to its amino acid residues before being able to incorporate it into its individual cell protein (65, 33, 87). Some may hold that present concepts of specificity of protein structure are incompatible with synthesis from large aggregates (polypeptides or greater), but we do not find sufficient knowledge of the nature and synthesis of protein in vivo to rule out such a reasonable hypothesis.

On the other hand, it must be admitted that an alternative hypothesis is tenable, namely, that intracellularly ingested plasma protein may be catabolized to amino acids at the same rate as normal cell protein and then 1, deaminized, sparing the cell protein, or 2, formed into new cell protein while catabolism of the old continues. No present evidence would bar the simultaneous intracellular catabolism and anabolism of such proteins at rates consistent with the maintenance of the normal tissue concentrations (78, 79) of amino acids.

Perhaps further speculation may be tolerated. It is admitted that the body can be kept in nitrogen equilibrium by amino acids by mouth. There will be synthesis of plasma proteins in the liver in this type of experiment and these plasma proteins will be available all over the body to all cells. In an emergency (fasting) these same plasma proteins can supply all the nitrogen (and protein) requirements. Why must we assume that the complicated assembly of amino acids to the large protein aggregate is the task of each and every cell in the body? It would be possible for the liver, strategically situated as it is, to do the hard work of preliminary synthesis to spare this task to the body cells specialized for other purposes (e.g., muscle). These specialized cells under standard conditions could rely upon the reservoir of plasma proteins for their current needs. This thesis may not be accepted by many but at least it fits with some evidence which is accumulating.

One may in *conclusion* present the following as a concept of plasma protein metabolism and its relation to total protein metabolism. The ultimate source of construction materials is food protein furnishing amino acids absorbed from the intestinal tract and synthesized in the liver cells (and elsewhere) into plasma proteins. This same influx of amino acids, and/or the plasma proteins, support protein formation throughout the body. A part of the body protein forms a *reserve against adversity* in the sense that it can be circumspectly depleted without apparent injury to the body. The supplies of amino acids coming from outside the body and the demand for protein materials within the body

are in a constant state of balance with these protein reserve stores, a *dynamic equilibrium.*

If the body need for protein to build new cell protein, new plasma protein, or new hemoglobin is greater than the exogenous supply, this reserve store of protein will be drawn upon; if the body need is less, the store will be repleted. The maximum and minimum limits of these stores are an individual characteristic of each organ, tissue, and fluid and are determined by factors not yet understood.

When the body demands protein the plasma contributes its share and evidence from plasma injection experiments indicates that plasma protein molecules can be accepted as such by body cells and recast into specific cell protein without loss of nitrogen.

It seems to us probable that new plasma protein flows largely from the liver although some globulins may be formed elsewhere.

REFERENCES

(1) Abel, J. J., L. G. Rowntree and B. B. Turner. J. Pharmacol. and Exper. Therap. **5**: 625, 1914.
(2) Addis, T. Proc. Soc. Exper. Biol. and Med. **40**: 336, 1939.
(3) Addis, T., L. J. Poo and W. Lew. J. Biol. Chem. **115**: 111, 1936.
(4) Addis, T., L. J. Poo and W. Lew. J. Biol. Chem. **115**: 117, 1936.
(5) Addis, T., L. J. Poo and W. Lew. J. Biol. Chem. **116**: 343, 1936.
(6) Alcock, R. S. Physiol. Rev. **16**: 1, 1936.
(7) Bennhold, H., E. Kylin and St. Ruszнyák. Die Eiweisskörper des Blutplasmas. Dresden und Leipzig, 1938, p. 41.
(8) Berg, W. Biochem. Ztschr. **61**: 428, 1914.
(9) Bergmann, M. Chem. Rev. **22**: 423, 1938.
(10) Bloomfield, A. L. J. Exper. Med. **59**: 687, 1934.
(11) Bock, H. E. Ztschr. ges. exper. Med. **82**: 539, 1932.
(12) von Bonsdorff, B., H. Groth and T. Packalén. F. Hematologica **59**: 184, 1938.
(13) Borsook, H. and G. L. Keighley. Proc. Roy. Soc. (London) **118B**: 488, 1935.
(14) Butt, H. R., A. M. Snell and A. Keys. Arch. Int. Med. **63**: 143, 1939.
(15) Canto, A. Compt. rend. Soc. de biol. **104**: 1103, 1930.
(16) Cathcart, E. P. The physiology of protein metabolism. London, 2nd ed., 1921.
(17) Chanutin, A., J. C. Hortenstine, W. S. Cole and S. Ludewig. J. Biol. Chem. **123**: 247, 1938.
(18) Corin, G. and G. Ansiaux. Vrtljschr. gerichtl. Med. 3. f. 7: 80, 1894.
(19) Cutting, W. C. and R. D. Cutter. Am. J. Physiol. **114**: 204, 1935.
(20) Daft, F. S., F. S. Robscheit-Robbins and G. H. Whipple. J. Biol. Chem. **103**: 495, 1937.
(21) Daft, F. S., F. S. Robscheit-Robbins and G. H. Whipple. J. Biol. Chem. **123**: 87, 1938.

(22) Doyon, M. Compt. rend. Soc. de biol. **57**: 30, 1905.
(23) Drury, D. R. and P. D. McMaster. J. Exper. Med. **50**: 569, 1929.
(24) Earle, I. P. and P. E. Howe. Science **77**: 213, 1933.
(25) Elman, R. Proc. Soc. Exper. Biol. and Med. **36**: 867, 1937.
(26) Elman, R. and D. O. Weiner. J. A. M. A. **112**: 796, 1939.
(27) Fiessinger, N. and S. Gothie. Compt. rend. Soc. de biol. **112**: 1053, 1933.
(28) Fischer, A. Science **75**: 443, 1932.
(29) Foster, D. P. and G. H. Whipple. Am. J. Physiol. **58**: 407, 1922.
(30) Goettsch, E. and E. B. Reeves. J. Clin. Investigation **15**: 173, 1936.
(31) Holman, R. L., E. B. Mahoney and G. H. Whipple. J. Exper. Med. **59**: 251, 1934.
(32) Holman, R. L., E. B. Mahoney and G. H. Whipple. J. Exper. Med. **59**: 269, 1934.
(33) Howland, J. W. and W. B. Hawkins. J. Biol. Chem. **123**: 99, 1938.
(34) Jameson, E. Proc. Soc. Exper. Biol. and Med. **36**: 808, 1937.
(35) Johansen, A. H. Acta Path. et Microbiol. Scand. supplement **37**: 272, 1938.
(36) Jones, T. B. and H. P. Smith. Am. J. Physiol. **94**: 144, 1930.
(37) Kerr, W. J., S. H. Hurwitz and G. H. Whipple. Am. J. Physiol. **47**: 356, 1918.
(38) Kerr, W. J., S. H. Hurwitz and G. H. Whipple. Am. J. Physiol. **47**: 370, 1918.
(39) Kerr, W. J., S. H. Hurwitz and G. H. Whipple. Am. J. Physiol. **47**: 379, 1918.
(40) Knutti, R. E., C. C. Erickson, S. C. Madden, P. E. Rekers and G. H. Whipple. J. Exper. Med. **65**: 455, 1937.
(41) Li, H. M. Chinese J. Physiol. **10**: 7, 1936.
(42) Liu, S. H. and H. I. Chu. J. Clin. Investigation **14**: 293, 1935.
(43) Liu, S. H., H. I. Chu, S. H. Wang and H. L. Chung. Chinese J. Physiol. **6**: 73, 1932.
(44) Loewi, O. Arch. exper. Path. und Pharmakol. **48**: 303, 1902.
(45) Luck, J. M. J. Biol. Chem. **115**: 491, 1936.
(46) Luck, J. M. The liver proteins in perspectives in biochemistry. Cambridge, 1937.
(47) Madden, S. C., P. M. Winslow, J. W. Howland and G. H. Whipple. J. Exper. Med. **65**: 431, 1937.
(48) Madden, S. C., W. E. George, G. S. Waraich and G. H. Whipple. J. Exper. Med. **67**: 675, 1938.
(49) Madden, S. C., W. A. Noehren, G. S. Waraich and G. H. Whipple. J. Exper. Med. **69**: 721, 1939.
(50) Madden, S. C., C. A. Finch, W. G. Swalbach and G. H. Whipple. To be published.
(51) Mann, F. C. Medicine **6**: 419, 1927.
(52) Mann, F. C. and J. L. Bollman. Proc. Staff Meet. Mayo Clinic **4**: 328, 1929.
(53) Mann, F. C., J. L. Bollman and J. Markowitz. Am. J. Physiol. (abstract) **90**: 445, 1929.
(54) McLean, F. C. Physiol. Rev. **18**: 495, 1938.
(55) McNaught, J. B., V. C. Scott, F. M. Woods and G. H. Whipple. J. Exper. Med. **63**: 277, 1936.

(56) MELNICK, D. AND G. R. COWGILL. J. Exper. Med. **66:** 493, 1937.
(57) MELNICK, D. AND G. R. COWGILL. J. Nutrition **13:** 401, 1937.
(58) MELNICK, D., G. R. COWGILL AND E. BURACK. J. Exper. Med. **64:** 877, 1936.
(59) MELNICK, D., G. R. COWGILL AND E. BURACK. J. Exper. Med. **64:** 897, 1936.
(60) MILELLA, A. Policlin. **42:** 220, 1935 (Chem. Abstr. **30:** 4551, 1936).
(61) MOLL, L. Beitr. chem. Physiol. und Path. **4:** 563, 1903.
(62) MORAWITZ, P. Beitr. chem. Physiol. und Path. **7:** 153, 1906.
(63) MOSCHOCOWITZ, E. J. A. M. A. **100:** 1086, 1933.
(64) NOLF, P. Arch. Internat. de Physiol. **3:** 1, 1905.
(65) POMMERENKE, W. T., H. B. SLAVIN, D. H. KARIHER AND G. H. WHIPPLE. J. Exper. Med. **61:** 261, 1935.
(66) POMMERENKE, W. T., H. B. SLAVIN, D. H. KARIHER AND G. H. WHIPPLE. J. Exper. Med. **61:** 283, 1935.
(67) REIMAN, H. A., G. MEDES AND L. FISHER. F. Hematologica **52:** 187, 1934.
(68) RITTENBERG, D., R. SCHOENHEIMER AND A. S. KESTON. J. Biol. Chem. **128:** 603, 1939.
(69) ROSE, W. C. Science **86:** 2231, 1937.
(70) ROSE, W. C. Physiol. Rev. **18:** 109, 1938.
(71) ROSE, W. C. AND E. E. RICE. Science **90:** 186, 1939.
(72) ROSENTHAL, F., H. LICHT AND E. MELCHIOR. Arch. exper. Path. und Pharmakol. **115:** 138, 1926.
(73) SABIN, F. R. J. Exper. Med. **70:** 67, 1939.
(74) SCHMIDT, C. L. A., editor. The chemistry of the amino acids and proteins. Springfield, Illinois, 1938.
(75) SCHULTZ, E. W., J. K. NICHOLES AND J. H. SCHAEFER. Am. J. Path. **1:** 101, 1925.
(76) SMITH, H. P., A. E. BELT AND G. H. WHIPPLE. Am. J. Physiol. **52:** 54, 1920.
(77) THOMPSON, W. H., I. MCQUARRIE AND E. T. BELL. J. Pediatrics **9:** 604, 1936.
(78) VAN SLYKE, D. D. J. Biol. Chem. **16:** 187, 1913.
(79) VAN SLYKE, D. D. AND G. M. MEYER. J. Biol. Chem. **16:** 231, 1913.
(80) WARNER, E. D. J. Exper. Med. **68:** 831, 1938.
(81) WARNER, E. D., K. M. BRINKHOUS AND H. P. SMITH. Am. J. Physiol. **114:** 667, 1936.
(82) WEDEKIND, T. Klin. Wchnschr. **12:** 103, 1933.
(83) WEECH, A. A. New York Acad. Med. Bull. **15:** 63, 1939.
(84) WEECH, A. A. AND E. GOETTSCH. Johns Hopkins Hosp. Bull. **63:** 154, 1938.
(85) WEECH, A. A. AND E. GOETTSCH. Johns Hopkins Hosp. Bull. **63:** 181, 1938.
(86) WEECH, A. A., E. GOETTSCH AND E. B. REEVES. J. Exper. Med. **61:** 299, 1935.
(87) WHIPPLE, G. H. Am. J. Med. Sci. **196:** 609, 1938.
(88) WHIPPLE, G. H. AND S. H. HURWITZ. J. Exper. Med. **13:** 136, 1911.
(89) WILLIAMS, R. D. AND A. B. GUTMAN. Proc. Soc. Exper. Biol. and Med. **34:** 91, 1936.
(90) WILLIAMSON, C. S., F. J. HECK AND F. C. MANN. Am. J. Physiol. (abstract) **59:** 487, 1922.
(91) Unpublished data.
(92) 31, 66, 56, 47, 48, 49, 50.

THE STUDY OF INTERMEDIARY METABOLISM OF ANIMALS WITH THE AID OF ISOTOPES

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The study of intermediary metabolism is concerned with synthesis, interconversion and degradation of organic molecules. In adult animals in energy equilibrium the total amount, the distribution and the chemical constitution of body constituents are kept constant within narrow limits. The constituents of normal diets (proteins, fats, carbohydrates, etc.) are in principle the same as those which compose the animal body. The experimenter loses track of them as soon as they pass the intestinal wall and mix with the same substances in blood and organs. Very few experiments, therefore, on the intermediary metabolism of physiological compounds have been carried out with adult animals on normal diets, as in most instances the tracing of the food constituents was impossible. Our present knowledge of intermediary metabolism is based mainly, therefore, on experiments performed under such abnormal conditions as permit accumulation of intermediates or end-products: Use of unnatural compounds, or of diets unduly enriched or poor in test-substances, experiments on poisoned, or sick organisms or on isolated organs, tissues or extracts.

The isotopes of the elements C, H, N, S, O, which constitute organic matter provide tools to follow organic molecules directly in the intact and normal animal. There are two types of isotopes available for biological application: 1, the natural stable isotopes of hydrogen (1), oxygen (2), nitrogen (3, 4), carbon (5), and sulphur (4), all of which have been made available by Urey and collaborators through fractionation of the natural elements; 2, a great number of artificially produced radioactive isotopes. In the present review, which is concerned with the metabolism of organic compounds, only those radioactive isotopes will be discussed which have been used as markers in organic molecules; namely, ${}_{15}P^{31}$ and ${}_{16}S^{35}$, etc. No mention will be made of the extensive work in which radioactive isotopes have been employed in the study of the metabolism of ions.

Hevesy, who has recently reviewed his extensive work on radiophosphorus (6), was the first to realize the value of isotopes as biological tracers. As early as 1923 (7) when neither stable nor radioactive isotopes of biological elements were available, he followed the course of lead in animals by adding to it a minute amount of one of its natural radioactive isotopes (RaD). When heavy water became available, he carried out the first and one of the most fundamental experiments on water metabolism (8). He found the average time a water molecule spends in the human organism to be 14 days.

Most of the work on intermediary metabolism employing isotopes has been carried out with heavy hydrogen and heavy nitrogen. One paper by Tarver and Schmidt (9) has appeared in which radioactive sulphur was used for the study of the biological conversion of methionine into cystine. The biological introduction of radioactive iodine into the thyroid gland has been investigated by Hertz, Roberts and Evans (10). Hahn, Bale, Lawrence and Whipple (11) have used radioactive iron as a tool for the study of hemoglobin formation. They found inorganic iron to be poorly absorbed by normal, but readily by anemic dogs. Radioactive carbon has been employed for the study of photosynthesis (Ruben, Hassid and Kamen, 12). The short half life time of radioactive carbon makes its application to the study of intermediary metabolism of animals very difficult. Extensive work has been carried out with radioactive phosphorus. Phosphorus is not a true component of organic molecules but is found as phosphoric acid combined in ester or amide-linkage with organic molecules. The use of this isotope has proved of great value in the study of biological phosphorylation. In this review, phosphorus will be discussed only insofar as its use has direct bearing on the metabolism of organic molecules.

General properties of isotopes. Isotopes of the same elements have properties so similar that they are not appreciably fractionated in nature and the abundance is therefore practically the same everywhere. Even deuterium, which of all isotopes shows the greatest difference from its light analogue, is not measurably fractionated when organic compounds are formed in animals or plants. Dole (13) has shown in careful studies that the abundance of deuterium in honey, cholesterol, and benzene is the same as in water (see also Breusch and Hofer (14) and Steward and Halcomb (15)). The abundance of N^{15} in amino acids and proteins is practically the same as that in air (16). Living cells thus cannot distinguish between the isotopes of the same elements, but treat them practically alike.

Even if the deuterium content of water be artificially increased, the living organism does not fractionate it. Krogh and Ussing (17) after giving animals heavy water to drink, found the deuterium content to be the same in the water of all organs, blood and urine.

This inability of the cells to distinguish between isotopes also applies to isotopic organic compounds. A normal monoamino acid is a mixture of molecules, 99.63 per cent of which contain only N^{14}, and 0.37 per cent contain only N^{15}. The equal distribution of N^{15} in air and protein is proof that the cell treats both molecular species alike. If a physiological compound be administered in which the concentration of the isotopes is increased, this cannot constitute a material foreign to the body, as the living cell is accustomed to molecules containing "heavy atoms."

In work with deuterium, however, care must be taken not to raise its concentration too much. It has been shown by several workers (18) that high concentrations of heavy water (above 25 per cent) in the physiological fluids have a toxic influence, probably due to the changes in physical properties of the medium in which the biochemical reactions occur. As it has been shown that the rates of enzymatic reactions are different in heavy water (19) (either faster or slower), the biological effect of high concentrations may be due to disorganization of the delicately balanced rates. Concentrations of D_2O in the medium up to 10 per cent have no detectable influence. Organic compounds in which a large part or all of the hydrogen atoms has been replaced by deuterium may possibly be metabolized at different rates. Erlenmeyer *et al.* (20) have shown that succinic acid d_2 is dehydrogenated enzymatically at a slower rate than its normal analogue, and acetyl choline d_3 (21) has a smaller physiological action than ordinary acetyl choline. While the organic chemist may be interested in compounds with a definite and high deuterium content, the physiologist should employ substances with as low an isotope content as possible.

Stability and exchangeability of isotopes in organic linkage. When deuterium is employed as a marker of the carbon chain it has to be introduced into the compound in such a way that it will not be lost from the molecule either when dissolved in water or when transported in the aqueous fluids of the body. The deuterium atoms must be directly attached to carbon atoms. From our knowledge of organic reactions, it is in many cases possible to predict which hydrogen atoms will exchange their places with those of the aqueous medium. The use of deuterium has confirmed (22) the prediction that the hydrogen atoms in carboxyl, hydroxyl, amino, and other polar groups are ex-

tremely labile and exchange with the hydrogen of the aqueous medium at an immeasurably rapid rate. Such active hydrogen atoms are of no interest in metabolic research. Besides these extremely fast reactions, there exist others with slower rates: The hydrogen, attached to carbon atoms adjacent to ketonic groups, exchanges, due to enolization (23), and in some special cases (literature on exchange reactions, see 24, 25) other polar groupings may labilize the hydrogen of adjacent carbon atoms. Such slowly exchangeable hydrogen has been called semi-labile (26, 27). In most instances, however, the hydrogen of methyl and methylene groups is stable and is not removable by treating the compounds even at high temperatures with acid or alkali. Biological tracer experiments with deuterium are in general limited to such compounds as contain stably bound hydrogen atoms. As it is impossible on the basis of our present knowledge to predict with certainty whether the hydrogen atoms in a given compound will prove stable or labile, it is necessary in all metabolic experiments to test the stability of deuterium in the compounds administered as well as in those subsequently isolated from the living organism. Only in exceptional cases is it possible to use compounds with "semi-labile" deuterium, namely, when the rate of exchange is slower than that of the conversion of the substance into another with stable deuterium (28).

There is no indication that hydrogen atoms stable *in vitro* are labile *in vivo*. No enzymes have been observed which merely labilize carbon-bound hydrogen atoms without further involving the substance in a chemical reaction. The claim that proteolytic enzymes "exchange" stable hydrogen atoms in amino acids (29) has not been confirmed (30). The enzymatic replacement of deuterium by hydrogen due to dehydrogenases, *e.g.*, of deutero-succinic acid in ordinary water (20) COOH—CHD—CHD—COOH is not an enzymatic exchange reaction but a chemical reaction, involving reversible dehydrogenation and hydrogenation of the system fumaric $\rightleftharpoons$ succinic acid. If stably bound deuterium of organic compounds is removed, or if deuterium from the medium is introduced into stable positions of organic compounds, this reaction is always due to chemical processes (26, 30, 31, 32, 33, 34).

In contrast to hydrogen, carbon-bound nitrogen atoms do not exchange. The carbon-nitrogen linkage in all biological compounds investigated so far (35, 36) has proved to be stable. The same was found for the sulfur in sulfates (37) and in cystine (9). A biological replacement of normal N (or S) atoms by their isotopes can only be due to chemical reactions.

The oxygen isotope, O^{18}, while highly valuable as a tool for the in-

vestigation of chemical reactions (38) has not been used as a biological tracer for organic compounds. The high chemical "activity" of oxygen-containing groups and the exchangeability of carbonyl oxygen with the oxygen of water, restricts the application of this marker. Aten and Hevesy (39) have studied the excretion of sulfate ions, marked with heavy oxygen, and Day and Sheel (40) have followed the fate of heavy oxygen after inhalation by animals.

GENERAL METHODS FOR FOLLOWING METABOLIC REACTIONS. *Procedure A.* The isotopic compound, prepared in the laboratory (or biologically) is administered to an animal. By determining the isotope content of organs or their constituents, it is in many cases possible to follow the transportation and degradation of the material fed. The isolation of highly purified substances may throw light on processes of interconversion of the carbon chain. If a deuterium-containing compound A is given and a compound B also containing deuterium is in turn isolated, the deuterium content in B may be taken as indication that B has been derived from A. This conclusion, however, is valid only if the deuterium content in the stable hydrogen of B is higher than that of the body fluids. The administration of deuterium compounds generally leads to the formation, due to their biological degradation, of some heavy water, which becomes equally distributed over all body fluids. In the course of chemical reactions (hydrogenations, condensations, etc.) small amounts of deuterium from this heavy water may in turn enter into organic molecules (see below). The concentration of deuterium introduced by such reactions, however, cannot be higher than that of the medium (body fluids) in which the reactions occur.

The nitrogen isotope is a marker of the nitrogenous group (amino, imino group, etc.) but not of the carbon chain. In work with amino acids and other nitrogenous compounds, the metabolic fate of the carbon chain and the amino group has to be considered separately. Both are known to follow different metabolic routes. If an amino acid is utilized in a biological reaction, either the carbon chain, or the amino group, or both, may be involved. It is therefore advantageous, for the study of nitrogenous compounds, to use two independent isotope markers, deuterium (or carbon if available) for the carbon chain as well as N^{15}. A complete study of sulfur-containing amino acids may require the use of even more independent labels in one substance.

Procedure B. The isotope is administered in inorganic form (hydrogen as heavy water, nitrogen as ammonia, phosphorus as phosphate, etc.). The presence of the isotope in organic linkage (deuterium in

stable positions) indicates the occurrence of chemical reactions. Heavy water as drinking water has been widely used for the study of the formation of fatty acids (26, 31, 41, 42), and cholesterol (26), as well as for the detection of chemical reactions in the amino acids of proteins (17, 33, 42, 43, 44). The method also has been used extensively by Bonhoeffer and collaborators (45) for the study of the formation of organic compounds in lower plants.

Only a few organic compounds have as yet been found which in the living animal do not take up stably bound deuterium from the heavy water present in the body fluids. The absence of deuterium can in many cases be taken as proof that the compound was neither formed nor involved in a chemical reaction affecting the carbon chain. Such substances must have entered the body as such with the diet and could not have been formed from other substances. The administration of heavy water is thus a convenient procedure to study "chemical inertia" of body constituents. Up to the present, only the fatty acids, linoleic and linolenic acid (46), and the amino acid, lysine (33), have been found to be inert in animals. Not all essential food constituents (for instance indispensable amino acids) show such inertia. It has been found (see below) that some of them are involved in continuous and reversible chemical reactions, such as deamination $\rightleftharpoons$ amination, a process which leads to the replacement of one stable hydrogen atom. Ammonia has been found to be utilized by animals to some extent (47, 48) and may thus be employed as a tool to follow amination processes as well as the formation of urinary urea. It has, furthermore, been shown to be a convenient tool for the study of protein metabolism in plants (49). Inorganic sulfates are utilized in the formation of organic sulfur compounds by plants (50) but not by animals (9, 51).

PREPARATION OF ISOTOPIC COMPOUNDS. The number of methods is too large to permit of detailed description and only those more general procedures will be mentioned which have been employed for compounds which have been used in biological research.

A. Deuterium compounds. 1. The simplest method is the hydrogenation of double bonds with deuterium gas. The method has been used for the preparation of the following compounds; propionic acid from acrylic acid (52), butyric acid from crotonic acid (52, 53), caproic acid from sorbic acid (52), stearic acid from linoleic acid (54), leucine from isopentenal diethylcetal and valine from the next lower homologue (55), homocystine and methionine from acetylene (56), ornithine from α-pyridone (57), and coprostanone from cholestenone (58).

2. Another procedure is based on the fact that some catalysts may under certain experimental conditions labilize carbon-bound hydrogen atoms. Many compounds when treated with hot concentrated D_2SO_4 exchange otherwise stable hydrogen atoms (59). A number of deuterium-containing fatty acids and amino acids have thus been prepared by this procedure (60, 61). The method introduces deuterium into fatty acids only at the α-carbon atom.

Another catalyst is active platinum (62, 63, 64, 65). A number of deutero-fatty acids, otherwise not easily obtainable, such as palmitic acid, lauric acid, capric, caprylic acid (61), and iso-caproic acid (66) have been prepared by heating the normal analogues in D_2O with platinum and a trace of alkali. The deuterium in these compounds seems to be equally distributed among the stable hydrogen atoms of the whole molecule.

3. An easy but wasteful procedure is the biological preparation. Animals, when given heavy water, or plants, when grown on heavy water, form a great number of deutero-substances. This method while theoretically unlimited is restricted in practice by the cost of heavy water. More economical is the biological conversion of one compound into another: deutero-oleic acid has been prepared by feeding mice with deutero-stearic acid (67).

4. Various other methods which might be applied also to the preparation of physiological substances have been reviewed by Erlenmeyer (68).

B. Nitrogenous compounds. The starting material is isotopic ammonia. The usual laboratory procedures for the synthesis were modified so as to furnish good yields calculated on the basis of the ammonia employed rather than on the carbon chain. In work with isotopic ammonia the value of the nitrogen is greater than even the most complex carbon chain. The common synthesis of amino acids by treating α-bromo acids with a large excess of ammonia is thus not practical.

Modifications of the phthalimide procedure of Gabriel (69) and of the catalytic reduction of α-keto acids in the presence of ammonia (Knoop and Oesterlin (70)) have proved of special value. The following compounds have been prepared: glycine, alanine, phenylalanine, tyrosine, norleucine, leucine, glutamic acid, aspartic acid (66), ornithine (57), α-amino-γ-phenylbutyric acid (34), sarcosine, creatine and creatinine (36).

Biological synthesis of N^{15} compounds is possible and occurred in all experiments when isotopic ammonia or isotopic amino acids were fed. The isotope content of the newly formed substances, however, is low.

C. Sulfur compounds. The synthesis of methionine from radioactive sulfur via benzyl mercaptan has been described by Tarver and Schmidt (9). Radioactive glutathione has been prepared biologically by growing yeast on a medium containing radioactive sulfate ions (50).

D. Phosphorus. Various radioactive phosphoric esters have been prepared, by Parnas (72) by means of enzymatic reactions, and one has been synthesized by laboratory methods by Chargaff (71).

PRINCIPLES OF ANALYSIS OF ISOTOPES IN ORGANIC COMPOUNDS. The analytical methods used for the tracing of biological compounds must be highly sensitive. The isotopic material administered in small quantities will mix with large amounts of the same (normal) material present in the animal, whereby the isotope is "diluted" by large amounts of the normal elements. The analysis of radioactive isotopes, while easier and more sensitive than that of stable isotopes, is not more accurate. The radioactivity may be measured by different devices, the most commonly used being the Geiger counter. The substance may be ashed or the activity of the organic material as such may be measured (73).

For deuterium analysis the compound is burned and the heavy water content of the water obtained is determined. The most common procedures employed are the determination of refractive index (74) or of density. Various procedures for density determination have been employed. The "falling drop" method (75, 76, 77) is, in the experience of the authors, the most practical; the submerged float method, while highly sensitive, is too laborious for routine purposes.

There are several inherent difficulties in the determination of deuterium by density or refractive index of water. Both methods are based on the measurement of second order differences which are highly sensitive to impurities. Furthermore, the methods are limited for routine purposes to samples of water of not less than 70 mgm. The measurement of heat-conductivity of hydrogen gas or water employed by Farkas and Farkas (78) and Bonhoeffer and collaborators (45) (see also 79) requires much smaller samples, but is useful only for high concentrations of deuterium. All these difficulties are overcome by the use of the mass spectrometer, which promises to become by far the most satisfactory method for the determination of deuterium in hydrogen.

Mass spectrometric analysis is the only practicable method for the precise determination of other stable isotopes in organic compounds. For the determination of N^{15}, the organic substance is subjected to the Kjeldahl procedure and gaseous nitrogen is obtained by treatment of

the resulting ammonium sulfate with alkaline hypobromite. The method requires about 1 mgm. of nitrogen. It is highly sensitive; the ratio of the abundance of the masses can be determined to within one per cent. The N^{15} concentration in normal nitrogen can thus be determined within 0.003 per cent (80). As the isotopic compounds administered to animals usually contain several atom per cent excess[1] of isotope, the analytical procedures used are good enough to trace the marker even when the compound is mixed with several hundred (or even a thousand) times the amount of its normal analogue.

Purification of isotopic compounds by "washing out" procedure. In biological work on conversion of an isotopic compound A into another one B, it is frequently necessary to prove that the isotope in B is not due to a contamination of B with A, which may not be detectable by other chemical or physical methods. Purification up to constant isotope content is not always sufficient as many impurities, especially those of homologues, are frequently not readily removable by crystallization or similar fractionating procedures. Proof that the isotope is actually present in B can be secured by the use of the "washing out" procedure, which is based on the fact that isotopic compounds cannot be separated from their normal analogues by ordinary laboratory procedures. To the crude isotopic compound B is added a large amount of normal nonisotopic A, and B is again separated from A by the usual procedures. B is still contaminated with the same proportion of A as before; the latter, however, has now the composition of the mixture of small amounts of isotopic A (which were present as impurity in B) and the large amounts of normal A added. The procedure does not remove A from B but replaces most of the contaminating isotopic A by normal A. The procedure was employed in work on the interconversion of fatty acids (67, 81, 82, 83), as the chemical separation of these compounds is almost always incomplete. It has also been employed in work with radioactive phosphorus (84) and in the purification of isotopic, optically active amino acids obtained from synthetic mixtures (85, 34).

THE METABOLISM OF FOOD AND BODY CONSTITUENTS. *A. Metab-*

[1] It is convenient to calculate the isotope concentrations in terms of "atom per cent excess": Glycine, containing 10 atom per cent N^{15} excess means that the nitrogen of the glycine molecules contains 10 per cent more N^{15} atoms than normal glycine, i.e., 10.37 per cent of all its nitrogen is N^{15}. The same terminology was used in work with deuterium: valeric acid with 10 atom per cent deuterium means that 10.02 per cent of all its hydrogen atoms are present in the form of D.

olism of fatty acids. The only isotope marker used thus far for fatty acids is deuterium. All natural fatty acids contain stable hydrogen atoms in $-CH_3$, $-CH_2$ or $-CH$ groups. In the early work on transportation of fats in the animal organism, linseed oil was used that had been partially reduced with deuterium gas. Such a deutero-fat represents an ill defined mixture of fatty acids, some of which may even belong to the elaidic series and should be employed with caution in experiments on intermediary fat metabolism. It is preferable to employ isotopic fatty acids of known structure.

1. *Transport and deposition of dietary fatty acids.* Animals given marked fatty acids rapidly and extensively deposit them in the fat tissues and in the fat of the internal organs (81, 86, 87, 88). The highest concentration is always found in the internal organs (86), particularly in the liver (Barret, Best and Ridout (88); Cavanagh and Raper (87)) indicating a high "activity" of fat metabolism in this organ. The isotopic fatty acids are found not only in the neutral fat but in the phospholipids (87). Rapid deposition was also found after administration of individual isotopic fatty acids (stearic acid (82), oleic acid (67), palmitic acid (83)). The phenomenon, however, is restricted to higher fatty acids and is not observed when lower acids, such as butyric and caproic acids, are administered (52). These are rapidly and completely degraded. When given in large quantities to animals with ketosis, deutero-butyric acid is partly excreted as deutero-β-hydroxybutyric acid in the urine (Morehouse (53)).

The extensive deposition of the higher fatty acids is observed even when the animals are held at constant weight. The proportion of dietary fat deposited seems to be almost independent of the amount administered. Even when the diet is low in fat (1 per cent), a considerable part is deposited. In all such experiments in which the animals do not gain weight, the fatty acids utilized for energy requirements must be a mixture of dietary and body fatty acids. As much as 45–50 per cent of the dietary fatty acid administered in such small quantities was recovered from the body (83, 86).

As fatty acids are present in animals not in the free form but in ester linkage, the presence of isotopic dietary acids in the body fats indicates replacement of tissue acids involving continuous formation of ester linkages in normal animals. There are two reactions possible which might lead to fatty acid replacement: 1, continuous breakdown into glycerol and fatty acids and resynthesis of the total fat molecule, or 2, continuous ester shifts. Both reactions are known from *in vitro*

experiments to be induced by lipase. The isotope method determines only end-results and cannot yet distinguish between the two reactions. Both reactions may occur *in vivo*.

2. *The source of liver fats.* Barret, Best and Ridout (88) have made a careful study of the source of liver fat under different experimental conditions. By feeding, in a preliminary period, deuterium-containing fats to mice, they "marked" the fat tissues of the animals by the deposition of dietary fats. When anterior pituitary extracts or carbon tetrachloride were then given, the deuterium content in the liver fats clearly indicated their origin from the depot fat. However, the fat accumulating in the liver on diets low in protein and lipotropic factors (choline) had a low deuterium content. The fat accumulated in the liver under these conditions was thus not derived from the depot but probably synthesized in the liver from dietary carbohydrates. The authors found no transfer of depot fat to the liver when the animals were placed on a protein-rich diet. On the latter diet, the deuterium content of the fat depots is reported to remain constant for considerable periods, an indication of the stability *in vivo* of deuterium present in fatty acids.

3. *Interconversions of fatty acids.* While the ability of animals to form fatty acids from carbohydrates or proteins had been definitely established, proof for interconversions of fatty acids was lacking. The classical balance experimentation was unable to decide whether a fatty acid newly formed was derived from another fatty acid or from a non-fatty substance. The deuterium technique has proved to be a valuable tool for the study of such processes. It has not only established the ability of animals to convert one fatty acid into another, but has demonstrated that such reactions occur continuously even when the total amount and properties of the body fats do not change.

a. Desaturation and saturation of fatty acids. After administration of deuterium-containing saturated fatty acids to mice, the unsaturated fraction contained deuterium indicating its origin from the saturated acid (84). Not only is oleic acid continuously formed from stearic acid, but palmitoleic acid, which is a normal constituent of rat fat (89), is continuously formed from palmitic acid (83). Linoleic and linolenic acid, however, are not formed by desaturation (see later).

The reverse process, saturation of unsaturated acids, has also been demonstrated in mice (67): the animals received deuterium-containing oleic acid prepared biologically. The stearic acid of these animals contained deuterium, indicating its origin from the deutero-oleic acid.

b. Shortening of fatty acid chain by two carbon atoms. Mice degrade deutero-stearic acid to palmitic acid (82). The degradation of palmitic acid to lower acids (lauric and myristic acids) was followed in rats (83). The reactions occur continuously in normal animals. The findings may be taken as a new support for the theory of Knoop and Dakin of one-sided β-oxidation of natural fatty acids in normal animals.

c. Elongation of fatty acids by two carbon atoms. The feeding of small amounts of deutero-palmitic acid to normal rats results in the formation of deutero-stearic acid (83). This establishes continuous occurrence of *direct* elongation of the chain of palmitic acid by two carbon atoms.

All these conversions observed must have occurred without first breaking down the fatty acids into smaller units, as intermediate disruption would have led to a loss of most of the deuterium from the molecule.

Aliphatic alcohols as intermediates in fatty acid metabolism. Cetyl alcohol ($C_{16}H_{34}O$) as well as octadecyl alcohol ($C_{18}H_{38}O$) had been found to be minor but normal constituents of feces (90). They are not products of bacterial reduction, but are continuously secreted into the intestinal lumen (91). The origin of the aliphatic alcohols has now been traced to the fatty acids (83): after the ingestion of small amounts of deutero-palmitic acid, the fecal alcohols contained a high concentration of deuterium. On the other hand, animals rapidly convert these alcohols into the corresponding fatty acids: Feeding deutero-cetyl alcohol results in the formation of deutero-stearic acid as well as deutero-palmitic acid; deutero-octadecyl alcohol gives rise to deutero-palmitic acid as well as deutero-stearic acid. These processes are so rapid that the amounts of deutero-acids deposited in the depot fats of the animals are practically the same whether the deuterium is administered in the form of acid or of alcohol. As all these reactions have been studied in normal animals on their normal diets, they must be normal events. The alcohols may represent intermediates in the process of elongation of carbon chains described above.

Synthesis of fatty acids from small molecular units. No experiments have yet been carried out by following directly the formation of fatty acids from isotopic sugars or amino acids. It is questionable whether deuterium can be employed for such experiments. The intermediates of sugar metabolism are compounds of low molecular weight, of which most or all of the hydrogen is exchangeable. Deuterium originally introduced into the molecules of sugars or amino acids would be trans-

ferred from the carbon chain of the compounds to the aqueous medium at the instant when such labile intermediates are formed. In the second step, the condensation of smaller molecules to form larger ones, only normal hydrogen from the body fluids would be introduced. The loss of deuterium in the course of such reactions was observed with deutero-propionic acid. This substance is known to form a corresponding amount of glucose in diabetic animals. Administration of deutero-propionic acid to a diabetic dog resulted in extra production of glucose, which did not contain deuterium (52).

The introduction into organic compounds of hydrogen from the aqueous medium in the course of condensations or during hydrogenations can be used as a general procedure to study chemical reactions in normal animals (reaction B, p. 220). If fatty acids are formed from sugars or amino acids in a medium of heavy water instead of ordinary water, large amounts of deuterium must enter the reaction and take up stable positions in the newly formed fatty acid molecules. In the course of direct interconversions of fatty acids described above, only small amounts of deuterium will enter the molecule, as all these reactions are limited to a small number of carbon atoms. The formation of fats from sugar or amino acids must lead to a fixation of much more deuterium. When mice (or rats) are given heavy water to drink, a continuous formation of deuterium-containing fatty acids has been observed (26, 31, 41, 42). The process goes on until the deuterium concentration (in atom per cent) in the saturated fatty acids is half of that in the medium (body fluids) in which they were formed (26, 46). On extending the time of the experiment, no further rise in deuterium concentration occurs. The fatty acids must thus be formed by a process by which at least 1 out of 2 hydrogen atoms in the fatty acids is derived from the water of the medium, while the other normal atoms must have been derived from the starting material. Only a synthesis obtained from smaller units, such as the intermediates of carbohydrate or protein metabolism can be responsible for this result.

All theories of fat formation from sugars formulate aldol condensations and Cannizzaro reactions as intermediary steps. Bonhoeffer and collaborators (92, 93) have shown that these two reactions, when carried out in a medium of heavy water, do not lead to an introduction of stably bound deuterium into the resulting compounds, even though water enters the Cannizzaro reaction. This might explain why only half of the hydrogen atoms in the newly formed fatty acids are derived from the water of the body fluids. They are probably introduced in the course of reduction.

The administration of heavy water can thus be used as a means to follow synthesis of fatty acids in animals. The process has been studied in adult mice of constant weight on a fat-free diet. The observed new formation of fatty acid must have been coupled with a simultaneous degradation of an equivalent amount of fatty acids, *i.e.*, the uptake of deuterium measures the molecular regeneration[2] of fatty acid molecules in normal animals.

It is clear that such reactions are dependent upon the general conditions under which the animals are kept. However, the experiments are reproducible. It was found that in adult mice, half of their fatty acids is regenerated in 5 to 9 days,[3] *i.e.*, in a period much shorter than had been generally anticipated.

The rate of uptake of deuterium into different fatty acid fractions and individual fatty acids has been studied. The saturated fatty acids, stearic acid, as well as palmitic acid, were found to be regenerated at approximately the same rate (26, 46). The regeneration of unsaturated acids proceeds at the same rate as that of the saturated acids; however, the total concentration of deuterium in the unsaturated fraction is lower than that of the saturated one. This is due to the fact that the highly unsaturated fatty acids are not regenerated.

The biological inertia of linoleic and linolenic acids. In contrast to all the other fatty acids investigated so far, linoleic and linolenic acids do not "take up" deuterium from the heavy water of the body fluids (46). This finding must be taken as proof that they were not formed either directly from small molecules nor from other fatty acids, such as stearic or oleic acid. These highly unsaturated acids must have been derived directly from the diet. This finding is in agreement with the results of earlier authors (95) who found these acids to be indispensable dietary constituents.

[2] Up to the present we have employed the term "turnover" for the process of simultaneous synthesis and destruction. We now prefer the word *regeneration*. "Turnover" implies the replacement of molecules by the same kind from any source, also by those of the diet. All biological substances, even inorganic ions are "turned over," but only certain organic compounds are regenerated, i.e., resynthesized after destruction. Hevesy in his work on isotopic phosphorus has used the word rejuvenation for the replacement of only part of a molecule, e.g., the replacement of phosphate in phospholipid or phosphocreatine by other phosphorus.

[3] It is a mistake to determine the time necessary for *complete* replacement of molecules by newly formed ones, i.e., the time necessary for *total regeneration*. As such reactions follow exponential curves, their time is infinite.

The metabolism of phospholipids. The most abundant phospholipids, lecithin and cephalin, are compounds of glycerol, fatty acids, phosphoric acid, and a base (choline or aminoethanol). Isotopes maybe used to study the metabolism of any of these components. While in a few cases, the uptake of deutero-fatty acids into phospholipids was studied (87), most of the work has been carried out with radioactive phosphorus. The first experiments with this tool were performed by Chievitz and Hevesy (96) and by Artom and collaborators (97, 98). Hevesy (6) has recently reviewed his extensive and important work.

Hahn, Hevesy and Lundsgaard (99) found by administration of radioactive phosphate that the average time an atom of phosphorus remains in the body of a rabbit is about 30 days. When radioactive phosphates are administered to animals, the phosphorus of the phospholipids displays radioactivity (6). Phosphorus in these compounds is linked to two alcohol groups, namely, that of glycerol and that of choline or cholamine. The appearance of new phosphorus can only be due to chemical reactions, not to mere exchange. It indicates either complete or partial breakdown followed by resynthesis of the phospholipids. As the reaction has been observed in normal animals, even when no fatty acids were given, phospholipids, like fats and fatty acids, must be continuously regenerated. Hevesy and Lundsgaard (100) fed fat and radioactive phosphorus to a dog in order to determine where the increased lecithin was synthesized. They conclude that the site of formation is not the intestinal tract. In another experiment, Hevesy and Hahn (10) injected biologically prepared radioactive phosphatide into the blood stream of a rabbit and found that one-half of it disappeared from the blood in 1.5 hours. After a lapse of three hours, one-third of the phosphatides was in the liver. Robinson and collaborators (102) showed that tissue slices of liver, kidney and intestine can incorporate inorganic phosphate into phospholipids. In the intact animal different organs have a different turnover of phospholipids. Artom, Sarzana and Segré (98), Perlman, Ruben and Chaikoff (103), and Fries, Ruben, Perlman and Chaikoff (104) (see also 84, 105), have investigated the rate of turnover of phospholipids in various organs of rats. These authors find that the phospholipids of the liver show the highest activity, the small intestine and kidneys slightly lower, while the lowest activity is found in the brain (106). Artom, Sarzana and Segré (98) have, on the basis of reasonable assumptions, investigated mathematically the rates of the replacement of

labeled phosphorus in phospholipids. Jones, Chaikoff and Lawrence (107) have studied the regeneration of phospholipids in tumors. The rate in this tissue varies but is roughly the same as that of the liver. Perlman and Chaikoff (108) found the addition of choline and betaine accelerates phospholipid metabolism in the liver, while cholesterol retards it. Lecithin and cephalin are regenerated at about the same rate (Chargaff (109)).

Hevesy and Hahn (110) have given radioactive phosphate to laying hens. They found the administered phosphorus in the yolk, albumen and shell of the egg. They conclude that the bulk of the phospholipids in the egg is synthesized in the liver and transported through the plasma to the ovaries. The corpuscles play no part in this transport. Extensive phospholipid formation was observed in the chick embryo during development but none in the yolk (6). Labeled phospholipid appears in the egg yolk within six hours after administration of phosphate (111).

Steroid metabolism. The regeneration of cholesterol has been studied in mice on a cholesterol-free diet (26) by giving the animals heavy water to drink. The process is slower than with fatty acids, the half time being about 15–25 days (as compared to 5–9 days for fatty acids in the same animals). The amount of deuterium (in atom per cent) introduced into cholesterol is the same as has been found for fatty acids; during cholesterol synthesis at least every second hydrogen atom is derived from the aqueous medium. This indicates that cholesterol is synthesized by condensation of small molecular units. No appreciable sterol regeneration was observed in the developing chicken egg.

The process of coprosterol formation in dogs and humans has been studied with deuterium (58, 28). Deutero-cholestenone and deutero-coprostanone, which reappear as deutero-coprosterol, are both probably biological intermediates. Cholic acid isolated from bile after feeding deutero-coprostanone (112) did not contain deuterium. The ketone is probably not a precursor of bile acids.

Carbohydrate metabolism. As mentioned earlier, no experiments have been carried out by administration of isotopic sugars. One paper on the uptake of deuterium from the body fluids into newly-formed liver glycogen has been published by Ussing (113). The amount of stably bound deuterium in the glycogen of rats fed with sugar and heavy water was much higher than could be accounted for by a process

by which glucose molecules are merely coupled with each other. It was suggested that the monohexose was first split into smaller units.

A number of experiments on the formation of sugars in lower plants, as well as on alcohol formation by yeast, have been published by Bonhoeffer and collaborators (45).

PROTEIN METABOLISM. The proteins are the most complex compounds of the cell, containing a very large number of functional groupings. Most metabolic work at present is essentially a study of the reactions of smaller units (amino acids) and their linkages. From the standpoint of metabolism, each amino acid has at least two parts, the carbon chain and the amino group. It is desirable to employ for the study of their metabolism two different isotopes, one for the carbon chain and one for the amino group. Isotopic nitrogen has been available for such studies for only three years (3). The investigation of protein metabolism with N^{15} is still in the first stages of development.

The use of deuterium as a marker for the carbon chain of amino acids is somewhat more restricted than was the case with fatty acids and cholesterol. Some of the carbon-bound hydrogen in a few amino acids is "semi-labile," *i.e.*, slowly exchangeable at elevated temperature (29, 27, 44, 17, 45). The exchange at high temperature is probably the result of such reactions as partial racemization, equilibrium processes (*e.g.*, glutamic acid $\rightleftharpoons$ pyrolidone carboxylic acid) and of the mobility of the hydrogen atoms ortho to the phenolic group in tyrosine (27). Deuterium present in such positions may be lost partly or even completely during hydrolysis of proteins. Amino acids marked with deuterium at such positions may be employed with the greatest care only. Except for glycine, in which all the carbon-bound hydrogen atoms are "semi-labile," all amino acids investigated so far contain some stable hydrogen atoms (not exchangeable even under the drastic conditions of protein hydrolysis), which, when replaced by deuterium, may be utilized as markers for the carbon chain.

The fate of dietary nitrogen. The nitrogen excreted by adult animals kept in energy and nitrogen equilibrium has generally been considered to represent mainly dietary nitrogen, together with a small amount originating from repair of "wear and tear." This theory of exogenous and endogenous nitrogen metabolism has been tested by adding small amounts of amino acids containing N^{15} to the ordinary stock diet of adult animals to constant weight. Up to the present three amino acids have been tested (dl-tyrosine (114), l(−)-leucine (85) and glycine (115).

In all experiments only small proportions of the isotopic nitrogen

were recovered in the excreta and in the non-protein nitrogen fraction, about half was found to be introduced into the body proteins. This distribution seems to be typical and even when the isotopic nitrogen was given as ammonium salt, a considerable part of the nitrogen was recovered from tissue proteins (47, 48).

As all animals maintained constant weight and total protein content, the introduction of dietary nitrogen must have been due to chemical reactions of the proteins which led neither to an increase of total protein nor to a change of its structure. Isotopic nitrogen in amino acids or ammonia thus provides a tool of value not only for tracing the fate of the particular compound given but for the study of chemical reactions of the body proteins.

Direct replacement of amino acids in proteins by dietary amino acids. Part of the new nitrogen in the body proteins is due to direct introduction of the marked dietary amino acid, *i.e.*, of the original carbon chain with the amino group still attached to it. The investigation of this reaction requires the presence of an isotope marker in the carbon chain. Ussing (116) fed a rat, previously fasted, with a protein hydrolysate into which stable deuterium had been introduced by racemization in D_2O at 170°. Most of the deuterium was, therefore, located at the α-carbon atom of the amino acids. The deuterium content of the proteins of the liver of the animal indicated that at least 10 per cent of the liver protein and 2.5 per cent of the muscle protein were "newly" formed in the course of three days.

This biological reaction has been more thoroughly investigated with l(−)-leucine containing deuterium attached to the carbon chain as well as N^{15} (85). After this compound had been fed to normal rats for three days, leucine was isolated from liver proteins and from the proteins of the rest of the body. Both preparations contained the two markers, indicating that carbon chain and amino group were introduced together. From the deuterium analysis it was calculated that at least 32 per cent of the carbon chain of the dietary leucine had been introduced as leucine into the proteins of the animals.

The corresponding values obtained when tyrosine or glycine were administered were similar but not as definite, as the data were calculated on the basis of isotopic nitrogen only. As will be seen later, the nitrogen becomes too easily detached to be of much value for tracing the carbon chain. The same applies to the deuterium atom attached to the α-carbon atom, which is removed simultaneously with the detachment of the amino group.

The replacement of amino acids in the protein by identical amino

acids of dietary origin requires two successive processes, the first of which must involve the opening, the second the closing, of at least two peptide linkages.

Continuous deamination and amination of the amino acids in proteins. Only a fraction of the new (dietary) nitrogen introduced into the proteins of animals is present in the form of the amino acid to which it was originally attached. Amino acids other than that administered contain considerable quantities of isotopic nitrogen. This finding is based on the isolation and isotope analysis of pure amino acid samples from the proteins of liver, intestinal wall or total carcass of animals given dl-tyrosine (114), l(−)-leucine (85), glycine (115) or ammonia (47, 48). The following amino acids were thereby found to have taken up nitrogen from others: glycine, tyrosine, leucine, glutamic acid, aspartic acid, proline, histidine, arginine. None was ever found in lysine.

As the nitrogen-carbon linkage is stable (35), the presence of isotopic nitrogen in the other amino acids must be due to chemical reactions involving both the compound that "yielded," and that which "accepted" the marked nitrogen. Two processes are conceivable which may be responsible for the result: 1, continuous destruction and resynthesis of total molecules, or 2, continuous deamination and reamination of amino acids. Reaction 1 may be responsible for the introduction of new nitrogen into dispensable amino acids but not into some indispensable compounds (the carbon skeleton of which the animal organism is unable to synthesize).

More detailed investigation of leucine (85) and histidine (117), both of which are indispensable, showed that they had accepted nitrogen from other amino acids. This must have been due to mere amino shift, without synthesis of the carbon chain. l(−)-Leucine containing the two isotopes was found to have yielded isotopic nitrogen and had accepted normal nitrogen without equivalent destruction of the deuterium-containing carbon chain. Histidine, isolated from animals given isotopic ammonia, was degraded in the laboratory to β-imidazolelactic acid. The isotope was found in the α-amino group but not in the ring. Deamination and amination had occurred, not synthesis.

The amino shift is responsible for the fixation in proteins of the bulk of the dietary nitrogen. The direct introduction of amino acids previously described plays only a minor rôle. In the 3-day experiment with leucine only 30 per cent of the new nitrogen in the protein was still attached to leucine, while the remaining 70 per cent was found in other

protein constituents. The corresponding values for dl-tyrosine were 25 per cent in tyrosine and 75 per cent in amino acids other than tyrosine.

The isotope technique thus reveals the occurrence of rapid amino shifts among the amino acids of proteins in animals. A number of biological reactions had already been formulated for free amino acids, and these might have been the cause of the results. All of them require the preliminary deamination of at least one of the reacting members to the corresponding keto acid. Reductive amination of such keto acids in the presence of ammonia liberated from other amino acids has been proposed by Knoop (118). Von Euler and collaborators (119) have demonstrated this enzymatic reaction to occur *in vitro* with ammonia and keto-glutaric acid. The enzyme is specific for glutamic acid. According to Braunstein and Kritzmann (120) and other workers, glutamic and aspartic acids can, by a process of direct "transamination," transfer their nitrogen to other α-keto acids, to form new amino acids. This process was found to be reversible, *i.e.*, keto-glutaric acid may form glutamic acid from other amino acids which are simultaneously converted into the corresponding α-keto acids. On the basis of these recent findings, glutamic and aspartic acids should play a central rôle in the utilization of ammonia (primarily involving fixation of nitrogen at the α-amino group) and in the transmission of nitrogen from the carbon chain of one amino acid to others.

The results obtained with N^{15} strongly support the theory that the dicarboxylic acids play a central rôle in protein metabolism. Whenever isotopic amino acids (85, 114, 115) or ammonia were given (47, 48), the isotope concentration in the dicarboxylic acids was much higher than in any other amino acid (except the one in which the isotope was administered). The concentration of isotope in glutamic acid was always somewhat higher than in aspartic acid.

The α-amino nitrogen is linked in the peptide to two carbon atoms. The process of amino shift, like that of direct introduction, requires the opening, at least temporarily, of peptide linkages for the liberation of the amino groups of the reacting amino acids.

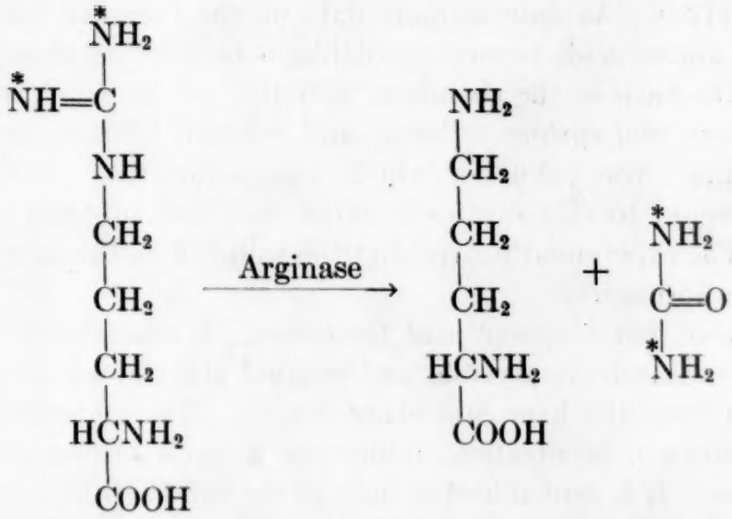
The "relative activity" of proteins of various organs and body fluids. As the total amount of proteins did not increase in the experimental animals, the fixation of dietary nitrogen must be due to continuous chemical processes which lead to no final quantitative or qualitative changes. The concentration of new nitrogen can be taken as a measure of relative "chemical activity" of the organ proteins.

Various organs have a different share in the "uptake" of dietary nitrogen. The serum protein always has the highest concentration of isotopic nitrogen, in the viscera (liver, intestinal wall, kidney, spleen, heart, testes, etc.) it is somewhat lower, while in muscle and skin it is lowest.

Although the concentration of "new nitrogen" in muscle and skin is very low, these two organs, by reason of their large size, play the most important rôle in the total replacement process. They usually contain more than 80 per cent of the total "new nitrogen" retained by the total proteins of the animals.

Deuterium may also be used to study chemical reactions in body proteins. Stekol and Hamill (43), also Krogh and Ussing (17, 42, 44), gave rats heavy water to drink and found stable deuterium in the body proteins. The latter authors found that electrical stimulation of muscles accelerates the introduction of deuterium. Stekol and Hamill hydrolyzed the proteins and found both tyrosine and cystine to contain stably bound deuterium. Foster, Rittenberg and Schoenheimer (33) repeated the experiments with two series of mice and isolated glycine, leucine, tyrosine, proline, arginine, histidine, glutamic acid, aspartic acid, cystine and lysine. All of these amino acids, with the exception of lysine, contained considerable amounts of stably bound deuterium. The authors concluded that this must be the result of chemical reactions, in which even indispensable amino acids (except lysine) take part and suggested the occurrence of continuous deamination and amination. Later experiments with N^{15} proved this suggestion to be true. It is of interest that in the experiments with deuterium, as well as with N^{15}, glutamic acid displayed the highest activity.

Activity of amidine group in arginine. The administration of isotopic amino acids or ammonia invariably results in the formation of isotopic arginine in the body proteins (47, 48, 85, 114, 115). Arginine has four nitrogen atoms, two in the ornithine moiety and two in the amidine group ($NH = C - NH_2$). The degradation of the biologically formed
|
isotopic arginine to urea (or ammonia) and ornithine showed that most of the isotope was present in the amidine group. Only in some cases was a small amount detected in the ornithine. According to the theory of urea formation through the ornithine cycle, proposed by Krebs and Henseleit (121), arginine is an intermediate in urea formation and the amidine group in arginine may be considered as "potential urea." The findings with N^{15} support this theory: Arginine is



The asterisks designate nitrogen atoms marked with isotope.

continuously degraded into urea and ornithine and the latter is reconverted into arginine. In the second stage of the process, isotopic nitrogen enters the amidine group. Arginase, however, supposed to be one of the enzymes involved in the process, reacts only slowly (122) or not at all with arginine in peptide or protein linkage. It is highly probable that the arginine isolated from the proteins was involved in urea formation during the frequent intervals when it was in the free state.

Continuous formation of arginine from ornithine and the replacement of protein-arginine by such newly-formed arginine have been directly demonstrated by employing deutero-ornithine, the feeding of which to adult mice of constant weight resulted in the presence of large amounts of deutero-arginine in the proteins of the animals (57).

The biological inertia of lysine. In contrast to all other amino acids hitherto investigated, lysine does not take up deuterium from the body fluids (33) nor N^{15} from other nitrogenous compounds (47, 85, 114).[4] Lysine may be degraded in the animal, but it is not involved in any other reactions affecting its carbon chain, such as synthesis or reversible amination. This finding is of interest in connection with the results of earlier workers who found that lysine, in contrast to many other indispensable amino acids, cannot be replaced in the diet of growing animals by the corresponding α-hydroxy acids (123), nor by its optical

[4] The amino acids investigated are: glycine, tyrosine, glutamic acid, aspartic acid, proline, cystine, histidine, arginine (also its ornithine moiety) and leucine.

antipode (124). As soon as more data on the chemical "activity" or inertia of amino acids become available, it may be possible to classify them on the basis of the chemical "activity" of their amino groups.

Methionine and cystine. Tarver and Schmidt (9) have synthesized dl-methionine, the sulfur of which was radioactive. After feeding this compound to rats cystine isolated from the proteins was radioactive. The experiment proves that the sulfur of methionine is utilized for cystine formation.

Glutathione and hippuric acid formation. Waelsch and Rittenberg (125) fed rats isotopic glycine, and isolated glutathione as the copper compound from the liver and other organs. The material contained a high isotope concentration, indicating a rapid regeneration of the glutathione. It is probable that most of the isotope in this peptide was present in the glycine moiety. The isotope content of the latter must then have been much higher than that of the glycine in the proteins of the corresponding organs, which was also isolated and analyzed. The simultaneous administration of benzoic acid, resulting in the excretion of isotopic hippuric acid, did not appreciably influence the isotope concentration in the glutathione. The experiments indicate that the replacement process in glutathione is much faster than in the proteins of the organs from which it had been isolated.

It had earlier been shown (126) that the administration to rats of isotopic glycine together with benzoic acid results in the excretion of hippuric acid, only part of which contains the isotopic tracer. A large amount of glycine in the hippuric acid must thus have been supplied from other sources, probably from proteins. The result may be taken as an indication that large amounts of glycine in proteins are available for detoxication and are so utilized, even when an abundant supply of "extra" glycine is administered.

Biological inversion of amino acids of "unnatural" steric configuration. The ability of animals to invert some amino acids of "unnatural" steric configuration into their "natural" isomers has been definitely established. Du Vigneaud and Irish (130) demonstrated that phenylaminobutyric acid, whether given as the "natural" or "unnatural" enantiomorph, is excreted in the urine as the acetyl product of the l form, the configuration of which corresponds to that of natural amino acids. Du Vigneaud and collaborators (34) have now followed this process, *i.e.*, inversion and acetylation with the aid of both N^{15} and deuterium. When the l-("natural") form with N^{15} in the amino group was given, the acetylated product in the urine contained almost the maximum amount of N^{15}, whereas the same substance isolated after the feed-

ing of the "unnatural" form contained only very little isotope. During inversion most of the nitrogen of the amino group must thus have been removed and replaced by nitrogen from other sources. When the reaction was studied with rats in which the body fluids contained heavy water, the phenylaminobutyric acid isolated from the urine contained one atom of stable deuterium which, by degradation of the amino acid, was proved to be attached to the α-carbon atom. This introduction of new hydrogen into the α-position was observed whether the "natural" or "unnatural" amino acid was administered. The results seem to indicate that acetylation of amino acids is connected with dehydrogenation of the amino acid, a reaction previously suggested by du Vigneaud and Irish (130). The experiment supports the view that biological inversion of an "unnatural" amino acid involves almost complete deamination to the corresponding keto-acid, followed by asymmetric amination.

THE METABOLISM OF CREATINE AND CREATININE. Most of the creatine in animals is present as phosphocreatine. Hevesy and Rebbe (94) have shown that the administration of marked phosphate results in a rapid formation of radioactive phosphocreatine in muscles of frogs. Three hours after injection, 78 per cent of the original phosphoric acid was replaced. The phosphocreatine thus shows a rapid regeneration. The same result is found when small amounts of creatine containing N^{15} are given to normal adult rats (36). Most of the isotope is then found in the creatine of muscles and internal organs, where it must have replaced creatine linked to phosphoric acid. The findings are in agreement with the concept that phosphocreatine is rapidly turned over in the course of muscle work.

The administration of isotopic creatine results in the excretion of isotopic creatinine in the urine, definitely establishing the conversion of creatine into creatinine (36). If no creatine is administered with the diet, body creatine is the only source of urinary creatinine. This result was obtained in experiments with rats which were given isotopic creatine in a preliminary period. As the result of the replacement of muscle and organ creatine by the isotopic dietary material, the animals then contained "marked" body creatine. In the subsequent periods, when the animals received a creatine-free diet, the urinary creatinine had the same isotope content as the body creatine.

The conversion of creatine into creatinine, reversible *in vitro*, is irreversible *in vivo*. When isotopic creatinine was administered, it was not converted into isotopic creatine but excreted unchanged.

By determining the rate of disappearance of isotopic creatine from

the muscles (by isotope analysis of urinary creatinine) in normal adult animals on a creatine-free diet, *i.e.*, when the total body creatine is constant, one measures the rate of regeneration of the creatine molecule proper. In contrast to all amino acids and most fatty acids, the creatine molecule was found to be only slowly regenerated: 2 per cent of the body creatine of adult animals was newly formed per day, which is about the same amount as is excreted in the urine as creatinine. There was no evidence in these experiments of any degradation of the creatine molecule: neither urinary ammonia nor urea contained isotope.

PHOSPHORYLATION IN MUSCLE. Radioactive phosphorus has proved to be a valuable tool for the study of such reactions. As stated above, the phosphate in phosphocreatine is replaced rapidly by inorganic phosphate (94) and the same was found for 2 of the 3 phosphates in adenosinetriphosphate by Meyerhof and collaborators (127). That the remaining phosphate is replaced very slowly was shown by Korzybski and Parnas (128), who injected radioactive phosphate into pigeons and rabbits and then isolated inosinic acid showing only slight radioactivity.

Some of the transfer reactions of phosphorus are direct and proceed without the intermediation of inorganic phosphate. Cozymase does not take up inorganic phosphate when involved in transfer. Even during the intramolecular rearrangement 3-phosphoglyceric acid $\rightleftharpoons$ 2-phosphoglyceric acid, inorganic phosphate does not enter the reaction (127). Similar results were obtained by Hevesy, Parnas and collaborators (129) who studied the formation of adenyltriphosphate from adenylic acid in the presence of isotopic phosphoglyceric acid: The adenyltriphosphate displayed a much higher radioactivity than the inorganic phosphate. The latter could thus not have been an intermediate.

ISOTOPIC COMPOUNDS AS INDICATORS FOR CHEMICAL REACTIONS OF BODY CONSTITUENTS. The results obtained by feeding isotopic physiological compounds to animals in energy and nitrogen equilibrium, *i.e.*, to animals of constant body composition, can scarcely be reconciled with the classical concept of independent exogenous and endogenous varieties of metabolism. The experiments with isotopic phosphates, fatty acids and amino acids have all shown that the body constituents are involved in continuous chemical processes and that there exists a close interaction between the food materials and the body components. Peptide, ester, and probably other linkages of the complex body ma-

terials, open and close continually. The amino acids, fatty acids or other units temporarily liberated, mix with others of the same species of whatever source, diet or tissue. By this mixing process they become indistinguishable as to their origin. While in the free state, the organic units take part in a variety of chemical reactions of which only few have as yet been investigated with isotopes; the amino acids are deaminated and may transfer their nitrogen to other deaminated molecules; fatty acids are interconverted into other types, and molecules newly derived from entirely different sources (*e.g.*, fatty acids from carbohydrates) enter this pool of liberated molecules. Parts of this metabolic mixture reënter vacant spaces in larger molecules (amino acids into proteins, fatty acids into fats, etc.). Phosphate ions continuously liberated from phospholipids and other organic phosphorus compounds mix with the relatively large amounts of phosphate ions in the body fluids and part of this mixture reënters the organic substance in the second stage of the process. As stated above, it is not at present possible to decide with certainty whether liberation and reintroduction of smaller units is associated with complete or only partial breakdown and resynthesis of larger molecules (fats, proteins). The findings of Bergmann (131) on enzymatic replacement of amino acids in peptides suggest that the observations with isotopes *in vivo* are also the result of replacement processes.

The nitrogen excreted in the urine may be regarded as part of the metabolic pool originating from the interaction of dietary nitrogen with the relatively large quantities of reactive body nitrogen, and the same applies to other atoms or molecular groupings in dietary and tissue substances (carbon dioxide in expiration, phosphates in urine, etc.). The rapid interaction of many substances makes the isotopic compound an indicator not only for the particular material administered, but for the large number of other molecules with which the compound interacts.

The fact that the living organism, in contrast to the dead material, keeps constant the form of the cells and organs as well as the chemical structure of the large molecules has led many investigators to believe that the tissue enzymes, which show their destructive power during autolysis, lie dormant during life and are "activated" only when their function is required. The results obtained with isotopes make such a supposition unnecessary. The experiments indicate that all reactions, for which specific enzymes and substrates exist in the animal, are carried out continually. The isotope method, therefore, in contrast to

others, does not require special experimental conditions for the investigation of metabolic processes. The normal adult animal on its normal diet is the best experimental subject.

REFERENCES

(1) Urey, H. C., F. G. Brickwedde and G. M. Murphy. Phys. Rev. **39:** 164, 1932; **40:** 1, 1932.

(2) Huffmann, J. R. and H. C. Urey. Ind. Eng. Chem. **29:** 531, 1937.
Lewis, G. N. and R. E. Cornish. J. Am. Chem. Soc. **55:** 2616, 1933.

(3) Urey, H. C., M. Fox, J. R. Huffmann and H. G. Thode. J. Am. Chem. Soc. **59:** 1407, 1937.
Thode, H. G. and H. C. Urey. J. Chem. Phys. **7:** 34, 1939.

(4) Thode, H. G., J. E. Gorham and H. C. Urey. J. Chem. Phys. **6:** 296, 1938.

(5) Roberts, I., H. G. Thode and H. C. Urey. J. Chem. Phys. **7:** 137, 1939.

(6) Hevesy, G. Enzymologia **5:** 138, 1938.
J. Chem. Soc. (London), 1213, 1939.

(7) Hevesy, G. Biochem. J. **17:** 439, 1923.
Biochem. Ztschr. **173:** 175, 1926.

(8) Hevesy, G. and E. Hofer. Klin. Wchnschr. **13:** 1524, 1934.

(9) Tarver, H. and C. L. A. Schmidt. J. Biol. Chem. **130:** 67, 1939.

(10) Hertz, S., A. Roberts and R. D. Evans. Proc. Soc. Exper. Biol. Med. **38:** 510, 1938.

(11) Hahn, P. F., W. F. Bale, E. O. Lawrence and G. H. Whipple. J. Exper. Med. **69:** 739, 1939.

(12) Ruben, S., W. Z. Hassid and M. D. Kamen. J. Am. Chem. Soc. **61:** 661, 1939.

(13) Dole, M. J. Am. Chem. Soc. **58:** 580; 2552, 1936.

(14) Breusch, F. and E. Hofer. Klin. Wchnschr. **13:** 1815, 1934.

(15) Stewart, W. W. and R. Holcomb. J. Am. Chem. Soc. **56:** 1422, 1934.

(16) Schoenheimer, R. and D. Rittenberg. J. Biol. Chem. **127:** 285, 1939.

(17) Krogh, A. and H. H. Ussing. Skand. Arch. Physiol. **75:** 90, 1936.

(18) Taylor, H. S., W. W. Swingle, H. Eyring and A. A. Frost. J. Chem. Phys. **1:** 751, 1933.
Lewis, G. N. J. Am. Chem. Soc. **55:** 3503, 1933.
Erlenmeyer, H. and F. Verzar. Ztschr. Biol. **97:** 519, 1936.
Ussing, H. H. Skand. Arch. Physiol. **72:** 192, 1935.
Barbour, H. G. and J. Trace. J. Pharmacol. **58:** 460, 1936.
Barbour, H. G. Yale J. Biol. Med. **9:** 551, 1937.
Craig, F. N. and S. F. Trelease. Am. J. Botany **24:** 232, 1937.

(19) Salzer, F. and K. F. Bonhoeffer. Ztschr. physik. Chem. **A 175:** 304, 1936.
Caldwell, M. L., S. E. Doebbeling and S. Manian. J. Am. Chem. Soc. **58:** 84, 1936.
Bonhoeffer, K. F. Ergebn. d. Enzymeforschung **6:** 47, 1937.
Thunberg, T. Enzymologia **3:** 56, 1937.

(20) Erlenmeyer, H., W. Schoenauer and H. Süllmann. Helv. chim. Acta **19:** 1376, 1936.

(21) Erlenmeyer, H. and H. Lobeck. Helv. chim. Acta **20:** 142, 1937.
(22) Bonhoeffer, K. F. and W. G. Brown. Ztschr. physik. Chem. **B 23:** 171, 1933.
(23) Klar, R. Ztschr. physik. Chem. **B 26:** 335, 1934.
Schwarz, K. and H. Steiner. Ztschr. physik. Chem. **B 25:** 153, 1934.
Halford, J. O., L. C. Anderson, J. R. Bates and R. D. Swisher. J. Am. Chem. Soc. **57:** 1663, 1935.
(24) Ingold, C. K. and C. K. Wilson. Ztschr. Elektrochem. **44:** 62, 1938.
(25) Koizumi, M. and T. Titani. Bull. of Chem. Soc. Japan, **13:** 318, 85, 95, 307, 298, 1938.
(26) Rittenberg, D. and R. Schoenheimer. J. Biol. Chem. **121:** 235, 1937.
(27) Rittenberg, D., A. S. Keston, R. Schoenheimer and G. L. Foster. J. Biol. Chem. **125:** 1, 1938.
(28) Anchel, M. and R. Schoenheimer. J. Biol. Chem. **125:** 23, 1938.
(29) Stekol, J. A. and W. H. Hamill. J. Biol. Chem. **120:** 531, 1937.
(30) Foster, G. L., A. S. Keston, D. Rittenberg and R. Schoenheimer. J. Biol. Chem. **124:** 159, 1938.
(31) Schoenheimer, R. and D. Rittenberg. J. Biol. Chem. **114:** 381, 1936.
(32) Schoenheimer, R. and D. Rittenberg. Science **87:** 221, 1938.
(33) Foster, G. L., D. Rittenberg and R. Schoenheimer. J. Biol. Chem. **125:** 13, 1938.
(34) du Vigneaud, V., M. Cohn, G. B. Brown, O. J. Irish, R. Schoenheimer and D. Rittenberg. J. Biol. Chem. **131:** 273, 1939.
(35) Keston, A. S., D. Rittenberg and R. Schoenheimer. J. Biol. Chem. **127:** 315, 1939.
(36) Bloch, K. and R. Schoenheimer. J. Biol. Chem. **131:** 111, 1939.
(37) Voge, H. H. and W. F. Libby. J. Am. Chem. Soc. **59:** 2474, 1937.
(38) Polanyi, M. and A. L. Szabo. Trans. Faraday Soc. **30:** 508, 1934.
Roberts, I. and H. C. Urey. J. Am. Chem. Soc. **60:** 880, 1938.
Mears, W. H. J. Chem. Phys. **6:** 295, 1938.
Roberts, I. J. Chem. Phys. **6:** 294, 1938.
Datta, S. C., J. N. E. Day and C. K. Ingold. J. Chem. Soc. 1968, 1937.
Cohn, M. and H. C. Urey. J. Am. Chem. Soc. **60:** 679, 1938.
Herbert, J. B. M. and I. Lauder. Trans. Faraday Soc. **34:** 432, 1938.
(39) Aten, A. H. W., Jr. and G. Hevesy. Nature **142:** 952, 1938.
(40) Day, J. N. E. and P. Sheel. Nature **142:** 917, 1938.
(41) Smith, P. K., J. Trace and H. G. Barbour. J. Biol. Chem. **116:** 371, 1936.
(42) Ussing, H. H. Skand. Arch. Physiol. **77:** 107, 1937; **78:** 225, 1938.
(43) Stekol, J. A. and W. H. Hamill. Proc. Soc. Exper. Biol. Med. **35:** 591, 1937.
(44) Krogh, A. and H. H. Ussing. Compt. rend. trav. lab. Carlsberg Sér. chim. **22:** 282, 1937.
(45) Reitz, O. and K. F. Bonhoeffer. Ztschr. physik. Chem. **A 172:** 369; **A 174:** 424, 1935.
Geib, K. H. and K. F. Bonhoeffer. Ztschr. physik. Chem. **A 175:** 459, 1936.
Salzer, F. and K. F. Bonhoeffer. Ztschr. physik. Chem. **A 176:** 202, 1936.

GÜNTHER, G. AND K. F. BONHOEFFER. Ztschr. physik. Chem. **A 180:** 185, 1937; **A 183:** 1, 1938.
(46) BERNHARD, K. AND R. SCHOENHEIMER. In press. J. Biol. Chem.
(47) FOSTER, G. L., R. SCHOENHEIMER AND D. RITTENBERG. J. Biol. Chem. **127:** 319, 1939.
(48) RITTENBERG, D., R. SCHOENHEIMER AND A. S. KESTON. J. Biol. Chem. **128:** 603, 1939.
(49) VICKERY, H. B., G. W. PUCHER, R. SCHOENHEIMER AND D. RITTENBERG. J. Biol. Chem. **129:** 791, 1939.
(50) FRANKLIN, R. G. Science **89:** 298, 1939.
(51) BORSOOK, H., G. KEIGHLEY, D. M. YOST AND E. MCMILLIAN. Science **86:** 525, 1937.
(52) RITTENBERG, D., R. SCHOENHEIMER AND E. A. EVANS, JR. J. Biol. Chem. **120:** 503, 1937.
(53) MOREHOUSE, M. G. J. Biol. Chem. **129:** 769, 1939.
(54) SCHOENHEIMER, R. AND D. RITTENBERG. J. Biol. Chem. **111:** 163, 1935.
(55) KINNEY, C. R. AND R. ADAMS. J. Am. Chem. Soc. **59:** 897, 1937.
(56) PATTERSON, W. I. AND V. DU VIGNEAUD. J. Biol. Chem. **123:** 327, 1938.
(57) CLUTTON, R. F., R. SCHOENHEIMER AND D. RITTENBERG. J. Biol. Chem. **132:** 227 (1940).
(58) SCHOENHEIMER, R., D. RITTENBERG AND M. GRAFF. J. Biol. Chem. **111:** 183, 1935.
(59) INGOLD, C. K., C. G. RAISIN AND C. L. WILSON. J. Chem. Soc. 915, 1643, 1936.
(60) SCHOENHEIMER, R., D. RITTENBERG AND A. S. KESTON. J. Am. Chem. Soc. **59:** 1765, 1937.
(61) VAN HEYNINGEN, W. E., D. RITTENBERG AND R. SCHOENHEIMER. J. Biol. Chem. **125:** 495, 1938.
(62) HORIUCHI, J., G. OGDEN AND M. POLANYI. Trans. Faraday Soc. **30:** 663, 1934.
(63) FARKAS, A., L. FARKAS AND E. K. RIDEAL. Proc. Roy. Soc. (London), **A 146:** 630, 1934.
(64) HORREX, C. AND M. POLANYI. Mem. Proc. Manchester Lit. Phil. Soc. **80:** 33, 1935–36.
(65) FARKAS, A. AND L. FARKAS. Trans. Faraday Soc. **33:** 678, 837, 1937.
J. Am. Chem. Soc. **60:** 22, 1938.
(66) SCHOENHEIMER, R. AND S. RATNER. J. Biol. Chem. **127:** 301, 1939.
(67) RITTENBERG, D. AND R. SCHOENHEIMER. J. Biol. Chem. **117:** 485, 1937.
(68) ERLENMEYER, H. Ztschr. Elektrochem. **44:** 8, 1938.
(69) GABRIEL, S. AND K. KROSEBERG. Ber. **22:** 426, 1889.
(70) KNOOP, F. AND H. OESTERLIN. Ztschr. physiol. Chem. **170:** 186, 1927.
(71) CHARGAFF, E. J. Am. Chem. Soc. **60:** 1700, 1938.
(72) PARNAS, J. K. Enzymologia **5:** 166, 1938.
(73) CHARGAFF, E. J. Biol. Chem. **128:** 579, 1939.
(74) CRIST, R. H., G. M. MURPHY AND H. C. UREY. J. Am. Chem. Soc. **55:** 5060, 1933.
J. Chem. Phys. **2:** 112, 1934.
(75) BARBOUR, H. G. AND W. F. HAMILTON. Am. J. Physiol. **69:** 654, 1924.
J. Biol. Chem. **69:** 625, 1926.

(76) Fenger-Eriksen, K., A. Krogh and H. Ussing. Biochem. J. **30:** 1264, 1936.
(77) Keston, A. S., D. Rittenberg and R. Schoenheimer. J. Biol. Chem. **122:** 227, 1937.
(78) Farkas, A. and L. Farkas. Proc. Roy. Soc. (London) A **144:** 467, 1934.
(79) Sachsse, H. and K. Bratzler. Ztschr. physik. Chem. A **171:** 331, 1934.
(80) Rittenberg, D., A. S. Keston, F. Rosebury and R. Schoenheimer. J. Biol. Chem. **127:** 291, 1939.
(81) Schoenheimer, R. and D. Rittenberg. J. Biol. Chem. **113:** 505, 1936.
(82) Schoenheimer, R. and D. Rittenberg. J. Biol. Chem. **120:** 155, 1937.
(83) Stetten, D., Jr. and R. Schoenheimer. J. Biol. Chem. (in press).
(84) Hahn, L. and G. Hevesy. Skand. Arch. Physiol. **77:** 148, 1937.
(85) Schoenheimer, R., S. Ratner and D. Rittenberg. J. Biol. Chem. **130:** 703, 1939.
(86) Schoenheimer, R. and D. Rittenberg. J. Biol. Chem. **111:** 175, 1935.
(87) Cavanagh, B. and H. S. Raper. Biochem. J. **33:** 17, 1939.
(88) Barrett, H. M., C. H. Best and J. H. Ridout. J. Physiol. **93:** 367, 1938.
(89) Klenk, E., F. Ditt and W. Diebold. Ztschr. physiol. Chem. **232:** 54, 1935.
(90) Gardner, J. A. Biochem. J. **15:** 244, 1921.
(91) Schoenheimer, R. and G. Hilgetag. J. Biol. Chem. **105:** 73, 1934.
(92) Fredenhagen, H. and K. F. Bonhoeffer. Ztschr. physik. Chem. A **181:** 379, 1938.
(93) Bonhoeffer, K. F. and W. D. Walters. Ztschr. physik. Chem. A **181:** 441, 1938.
(94) Hevesy, G. and O. Rebbe. Nature **141:** 1097, 1938.
(95) Burr, G. O. and M. M. Burr. J. Biol. Chem. **86:** 587, 1930.
(96) Chievitz, O. and G. Hevesy. Nature **136:** 754, 1935.
Danske Videnskab. Selskab. Biol. Medd. **13:** 9, 1937.
(97) Artom, C., G. Sarzana, C. Persier, M. Santangelo and E. Segré. Nature **139:** 836, 1105, 1937.
(98) Artom, C., G. Sarzana and E. Segré. Arch. Intern. Physiol. **47:** 245, 1938.
(99) Hahn, L. A., G. Hevesy and E. Lundsgaard. Biochem. J. **31:** 1705, 1937.
(100) Hevesy, G. and E. Lundsgaard. Nature **140:** 275, 1937.
(101) Hahn, L. and G. Hevesy. Nature **144:** 204, 1939.
(102) Robinson, A., I. Perlman, S. Ruben and I. L. Chaikoff. Nature **141:** 119, 1938.
(103) Perlman, I., S. Ruben and I. L. Chaikoff. J. Biol. Chem. **122:** 169, 1937.
(104) Fries, B. A., S. Ruben, I. Perlman and I. L. Chaikoff. J. Biol. Chem. **123:** 587, 1938.
(105) Cohn, W. E. and D. M. Greenberg. J. Biol. Chem. **123:** 185, 1938.
(106) Changus, G. W., I. L. Chaikoff and S. Ruben. J. Biol. Chem. **126:** 493, 1938.
(107) Jones, H. B., I. L. Chaikoff and J. H. Lawrence. J. Biol. Chem. **128:** 631, 1939.

(108) PERLMAN, I. AND I. L. CHAIKOFF. J. Biol. Chem. **127**: 211; **128**: 735; **130**: 593, 1939.
(109) CHARGAFF, E. J. Biol. Chem. **128**: 587, 1939.
(110) HEVESY, G. AND L. HAHN. Danske Videnskab. Selskab. Biol. Medd. **14**: 3, 1938.
(111) ENTENMAN, C., S. RUBEN, I. PERLMAN, F. W. LORENZ AND I. L. CHAIKOFF. J. Biol. Chem. **124**: 795, 1938.
(112) SCHOENHEIMER, R., D. RITTENBERG, B. N. BERG AND L. ROUSSELOT. J. Biol. Chem. **115**: 635, 1936.
(113) USSING, H. H. Skand. Arch. Physiol. **77**: 85, 1937.
(114) SCHOENHEIMER, R., S. RATNER AND D. RITTENBERG. J. Biol. Chem. **127**: 333, 1939.
(115) RATNER, S., D. RITTENBERG, A. S. KESTON AND R. SCHOENHEIMER. J. Biol. Chem. (in press).
(116) USSING, H. H. Nature **142**: 399, 1938.
(117) SCHOENHEIMER, R., D. RITTENBERG AND A. S. KESTON. J. Biol. Chem. **127**: 385, 1939.
(118) KNOOP, F. Ztschr. physiol. Chem. **67**: 489, 1910.
KNOOP, F. AND E. KERTESS. Ztschr. physiol. Chem. **71**: 252, 1911.
KNOOP, F. AND J. G. BLANCO. Ztschr. physiol. Chem. **146**: 267, 1925.
(119) VON EULER, H., E. ADLER, G. GÜNTHER AND N. B. DAZ. Ztschr. physiol. Chem. **254**: 61, 1938.
(120) BRAUNSTEIN, D. F. AND M. G. KRITZMANN. Enzymologia **2**: 129, 1937.
(121) KREBS, H. A. AND H. HENSELEIT. Ztschr. physiol. Chem. **210**: 33, 1932.
(122) KRAUS-RAGINS, I. J. Biol. Chem. **123**: 761, 1938.
(123) MCGINTY, D. A., H. B. LEWIS AND C. S. MARVEL. J. Biol. Chem. **62**: 75, 1924–25.
(124) BERG, C. P. J. Nutrition **12**: 671, 1936.
(125) WAELSCH, H. AND D. RITTENBERG. Science **90**: 423, 1939.
(126) RITTENBERG, D. AND R. SCHOENHEIMER. J. Biol. Chem. **127**: 329, 1939.
(127) MEYERHOF, O., P. OHLMEYER, W. GENTNER AND H. MAIER-LEIBNITZ. Biochem. Ztschr. **298**: 396, 1938.
(128) KORZYBSKI, T. AND J. K. PARNAS. Ztschr. physiol. Chem. **255**: 195, 1938.
(129) HEVESY, G., T. BARANOWSKI, A. J. GUTHKE, P. OSTERN AND J. K. PARNAS. Acta Biol. éxp. **12**: 34, 1938.
(130) DU VIGNEAUD, V. AND O. J. IRISH. J. Biol. Chem. **122**: 349, 1938.
(131) BEHRENS, O. K. AND M. BERGMANN. J. Biol. Chem. **129**: 587, 1939.

RELATION OF NICOTINIC ACID TO PELLAGRA

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Not infrequently a single observation may unite two apparently unrelated fields of scientific endeavor. The resulting relationship often increases the rate of progress in both fields. An excellent example of such mutual assistance is the recent progress in the fields of respiratory enzymes and vitamins. The work on cocarboxylase was given a definite stimulus when it was found to be related to vitamin B_1, the chemistry of which had taken years to elucidate. A similar relationship exists between the vitamin, riboflavin, and the yellow respiratory enzyme. Studies on the constitution of pyridine coenzymes undoubtedly aided in the identification of the antipellagra factor.

It is the purpose of this review to summarize briefly both the nutrition work and the enzyme work which led to the recognition of nicotinic acid as the antipellagra factor and then to discuss the significance of this relationship.

Pellagra was first recognized in Spain two centuries ago by Casal. Stannus and Gibson (1934) have reviewed the early observations on this disease. In 1830 the census in northern Italy revealed that in many areas 5 per cent of the population were suffering from pellagra. The disease was also endemic throughout France from 1818 to 1880, but after the latter date the disease is said to have practically disappeared. A large incidence has also been reported in the Balkan States. The disease is considered rather rare in Germany, Holland, Belgium and Great Britain.

During the past fifty years pellagra has been a definite problem in the United States. The first cases were recognized in 1863 by Gray in New York and Tyler in Massachusetts. A few sporadic cases were reported until 1907 when several were discovered in the mental asylums of South Carolina and Alabama. Voegtlin (1919–20) has reviewed the early studies on pellagra in the United States. Up to 1912, 30,000 cases with a fatality rate of 40 per cent had occurred. Clinical studies were started in Savannah, Georgia, shortly thereafter and the Thompson-McFadden Commission began its work in South Carolina in 1912.

Voegtlin postulated in 1914 that there was a causal relation between a mainly vegetable diet and pellagra and that a restricted vegetable diet might be defective on account of 1, a deficiency or absence of certain vitamins; 2, the presence of some toxic substance, and 3, a deficiency of certain essential amino acids. Goldberger, Waring, and Willets (1915) were the first to show conclusively that pellagra can be prevented by means of an appropriate diet. Three southern institutions were selected in which a large number of cases of pellagra had occurred for several years. In the fall of 1914 the diets were supplemented with milk, fresh meat, eggs, and dried beans. One year after the change only one out of 244 pellagrins had a recurrence of the disease whereas on the basis of previous experience a recurrence of about 50 per cent was expected. Goldberger and Wheeler (1915) produced typical pellagra in prison volunteers by placing them on a restricted diet.

It is beyond the scope of this review to describe in detail the symptoms of pellagra. The early symptoms are weakness, lassitude, anorexia and indigestion, followed by sore and ulcerated mouth and diarrhea. The typical dermatitis usually makes the diagnosis easy. According to Smith and Ruffin (1937) the patient may present in addition to the typical dermatitis on the exposed surfaces, seborrhea over the face and neck, hyperkeratosis over the bony prominences of the body, and lesions about the genitalia. Nervous manifestations occur in many cases of pellagra. Lorenz (1916) concluded that the psychosis accompanying pellagra has the characteristics of toxic psychoses in 90 per cent of the uncomplicated cases. The relation of sunlight to clinical manifestations of pellagra has been thoroughly reviewed by Smith and Ruffin (1937). The high incidence of pellagra during May to July is explained on the basis that there is a gradual accumulating deficiency during the winter months and when the patients become exposed to increasing intensity of the sunlight the acute symptoms of pellagra appear.

Sebrell (1938) has recently discussed the diet in the treatment of pellagra and points out that in the mild cases careful feeding is all that is necessary, but emphasizes that the severe cases require more energetic treatment. Spies (1935), however, demonstrated that the mortality rate among patients with severe pellagra, the majority of whom were addicted to alcohol, could be reduced from 54 per cent to 6 per cent in a series of 125 cases. McLester (1934) maintained that the death rate from severe endemic pellagra remained high irrespective of the method

of treatment. Later work by Spies, Chinn and McLester (1937) showed that endemic pellagra, like so-called alcoholic pellagra, responds to the administration of a high caloric, high protein diet, large amounts of yeast, and adequate rest.

The first report on the value of liver extract in pellagra was that of Ramsdell and Magness (1933) who found rapid clinical improvement in two cases upon the daily injection of 2 cc. of extract. The importance of liver extract was not accepted by all workers but the evidence (reviewed by Sebrell, 1938) was soon sufficient to warrant its use in severe cases of pellagra. The finding that liver extract had antipellagra activity gave considerable impetus to further animal work because liver extract had also been found to be effective in black tongue by Goldberger and Sebrell (1930).

Voegtlin (1919–20) described his early experiments with animals in which it was demonstrated that it was possible to produce in animals on a restricted vegetable diet both histological and chemical changes which in all respects except skin lesions were identical with those found in pellagra. Later Chittenden and Underhill (1917) found that dogs fed on a diet of crackers made from milled wheat flour, boiled peas, and vegetable fat developed diarrhea, loss of appetite and ulceration of the oral mucous membranes. The symptoms promptly disappeared when fresh meat was supplied. Wheeler, Goldberger, and Blacklock (1922) concluded that the condition was identical with canine black tongue, a disease in dogs so named because of the necrotic areas on the tongue; and later Goldberger and Wheeler (1928) were able to produce black tongue in dogs on diets similar to those eaten by humans in pellagrous areas. Goldberger and associates (reviewed by Sebrell, 1938) continued to collect evidence that substances capable of curing black tongue in dogs were equally effective in human therapy and vice versa. The two diseases were considered to be analogous and the term pellagra-preventive or black tongue-preventive factor was applied to the factor originally postulated by Goldberger and Tanner (1925). Since the activity in yeast was stable to autoclaving, the factor was associated with the more heat stable fraction of the B complex. However, Underhill and Mendel (1928) found that the condition produced in their dogs was cured by carotene and carotene-rich substances. This discrepancy has now been explained by Smith, Persons and Harvey (1937) on the basis that the oral lesions in both the Goldberger and Underhill types of canine black tongue are the results of infection with the fusospirochetal group of organisms secondary to lowered tissue resistance.

McCollum, Simmonds and Parsons (1919) tested the diet used by Goldberger and Wheeler (1915) on growing rats and found that the rats were able to live for many months but showed no increase in weight. Goldberger and Lillie (1926) produced a pellagra-like condition in rats on a diet deficient in the B complex but supplemented with an alcoholic extract of corn. They concluded that although it was highly probable that the pellagra-like condition in the rat was the analogue of pellagra in man, additional evidence was necessary to establish the relationship beyond doubt. The difficulties encountered in producing a deficiency of the antipellagra factor in rats is now well known and some of the problems encountered in attempting to use the rat for assay of this factor have been reviewed by Sebrell (1938) and Elvehjem (1938).

Guha (1931) found that liver extract 343 produced good growth in rats on a vitamin B_2 deficient diet and Salmon and Guerrant (1931) showed that liver extract contained four times as much vitamin G (B_2) as a sample of brewer's yeast. We now know that the growth obtained on liver extract in the case of rats was not due to the antipellagra factor but to other members of the B complex. However, the fractionation work on liver extract which followed not only aided in the identification of the antipellagra factor but other factors as well. In this connection it is interesting to note that the studies of Voegtlin, Neill and Hunter (1920) on the prophylactic value of foods in the treatment of pellagrins showed that the administration of yeast and rice extracts over a considerable period of time in large amounts failed to modify the course of the disease, with the possible exception of one case, but the administration of liver preparations was followed by a definite improvement.

Due to the difficulties encountered in the production of pellagra-like lesions in rats, Elvehjem and Koehn (1935) used chicks for assay of their concentrates. Chicks placed on a heated grain ration develop a typical dermatitis which is readily cured by low levels of liver extract. Although it is now established that the activity of liver extract in this syndrome is not due to nicotinic acid but rather pantothenic acid (Woolley, Waisman and Elvehjem, 1939; and Jukes, 1939), the work with chicks did give a new approach to the problem. It was soon found that the factor active for the chick was separate from riboflavin, which Kuhn, György, and Wagner-Jauregg (1933) had just isolated and shown to have growth-promoting properties in rats. Concentrates rich in riboflavin were completely inactive for the chick while purified fractions

from liver retained their potency after removal of riboflavin. Lepkovsky and Jukes (1935) also found riboflavin inactive and introduced the name "filtrate factor" for the active substance in the filtrate after removal of the riboflavin with fuller's earth.

Koehn and Elvehjem (1936) repeated the work they had done with chicks using dogs, since they recognized that there was no evidence to show that the pellagra-like condition obtained in chicks was identical with human pellagra. Black tongue was produced in dogs on a modified Goldberger diet. Concentrates of riboflavin from liver extract were found to be completely inactive in the prevention or cure of black tongue, but fractions from which the riboflavin had been removed and which were shown to be potent for chicks were highly active. Birch, György and Harris (1935) also found that the addition of riboflavin had no curative action on dogs that had developed black tongue on Goldberger's diet. Sebrell, Hunt and Onstott (1937) obtained similar results through the use of more carefully controlled conditions and relatively large doses of pure riboflavin. The complete inactivity of riboflavin in the treatment of human pellagra has been reported by Dann (1936) and Fouts, Lepkovsky, Helmer and Jukes (1936). Previous to the above studies Rhoads and Miller (1935) reported that it was impossible to produce black tongue in dogs on a diet low in vitamin G (as measured by rats) and suspected that black tongue was due to the lack of some other factor than vitamin G.

Further purification of the liver fractions gave concentrates which contained very small amounts of solid matter and showed high activity in both chicks and dogs and finally Elvehjem, Madden, Woolley and Strong (1937) demonstrated the activity of nicotinic acid in the cure of black tongue and isolated nicotinic acid amide from the concentrates. The activity of nicotinic acid in the treatment of black tongue was soon verified by Street and Cowgill (1937), Dann (1937), Sebrell and coworkers (1938), and Ruffin, Margolis, Margolis, Smith and Smith (1938). When the antipellagra factor became available in pure form, it was possible to clearly differentiate it from the chick antidermatitis factor. Fouts, Helmer, Lepkovsky, and Jukes (1937), Dann (1937), and Mickelsen, Waisman and Elvehjem (1938) have shown that nicotinic acid or the amide are completely inactive in the prevention of chick dermatitis.

It is interesting, now that the chick factor and the antipellagra factor have been differentiated, to look back upon some of the difficulties encountered. In their early work Lepkovsky and Jukes (1935) were inclined to believe that the material in the fuller's earth adsorbate was

the antipellagra factor because Goldberger, Wheeler, Lillie and Rogers (1928) had found the antipellagra activity in a fuller's earth adsorbate of an aqueous extract of yeast. Later Fouts, Lepkovsky, Helmer and Jukes (1936) cured pellagra with liver filtrate from which riboflavin and vitamin B_6 had been removed by adsorption on fuller's earth. Nicotinamide is not readily adsorbed on fuller's earth and the activity obtained by Goldberger and co-workers must have been due to the adsorption of nicotinamide along with other inert material. Jukes (1937) differentiated the chick factor and the antipellagra factor on the basis of the differences between the distribution of the two factors in foods. An important difference was in the case of wheat germ which was low in the chick factor and supposedly high in the antipellagra factor. Yet in more recent studies wheat germ has also been found to be low in nicotinic acid. Koehn and Elvehjem were fairly well convinced that the chick factor and the antipellagra factor were identical until the actual isolation of nicotinic acid. It is easy to see how this was possible when the chemical properties of the two compounds are compared. A preparation of pantothenic acid free of nicotinic acid is very hard to prepare.

The recognition of nicotinic acid as the antipellagra factor did not result without some help from the earlier studies on nicotinic acid. Huber (1867) first prepared nicotinic acid in 1867 by the oxidation of nicotine. Its isolation from biological material was not achieved until 45 years later when Funk (1913) isolated nicotinic acid in crystalline form from yeast concentrates which possessed antineuritic activity. The acid itself, however, displayed no activity in curing pigeon beriberi. At about the same time Suzuki, Shimamura and Odake (1912) isolated nicotinic acid from rice polishings. Williams (1917) impressed by the common occurrence of nicotinic acid with the antineuritic vitamin in several natural substances again tried nicotinic acid, trigonelline, as well as other pyridine derivatives for antineuritic potency, but none of them caused any permanent improvement in polyneuritic fowl. Trigonelline, the methyl betaine of nicotinic acid, was isolated from plant material by Jahns (1885). Ackerman (1912) found that dogs given fairly large amounts of nicotinic acid excreted in the urine about equal amounts of trigonelline and nicotinuric acid (the dipeptide of nicotinic acid and glycine).

Except for the work of Szymanska and Funk (1926), who attributed an appetite-stimulating and weight-preserving action to nicotinic acid

and the amide, very little interest was shown in the possible rôle of pyridine derivatives in living systems until the work of Warburg and von Euler in 1935. Warburg and Christian (1935) characterized nicotinic acid amide as one of the hydrolysis products from the coenzyme which they had isolated from blood and which is now known as coenzyme II. Kuhn and Vetter (1935) isolated nicotinic acid amide from heart muscle and von Euler, Albers and Schlenk (1935) from cozymase.

This work gave new impetus to the application of these compounds in the field of nutrition. Von Euler and Malmberg (1936), using a diet similar to the Sherman-Bourquin diet supplemented with thiamin and riboflavin, found no growth response with the acid or the amide although the rats receiving the acid lived longer. Funk and Funk (1937) found larger food intake and better growth in rats and pigeons on certain rations when given the acid and especially the amide. Frost and Elvehjem (1937) observed a growth stimulus from nicotinic acid when fed with adenylic acid to rats on a factor W deficient diet. However, none of these responses was of sufficient magnitude to attribute to nicotinic acid real vitamin-like properties.

The significance of nicotinic acid in the nutrition of microörganisms was also recognized at about this time. Knight (1937) showed that nicotinic acid was an essential growth factor for Staphylococcus aureus and that nicotinic acid was present in most active preparations of the Staphylococcus growth factor. Mueller (1937) showed that nicotinic acid is essential for diphtheria bacillus; Koser, Dorfman and Saunders (1938) for dysentery bacillus; and Fildes (1938) for Proteus.

The activity of nicotinic acid in the treatment of black tongue suggested its therapeutic use in human pellagra and the first report of its successful use was made by Spies, Cooper and Blankenhorn, and by Fouts before the Central Society for Clinical Research in Chicago, November 5, 1937. Published description of the work followed in rapid order (Fouts, Helmer, Lepkovsky and Jukes, 1937; Harris, 1937; Smith, Ruffin and Smith, 1937; Spies, Cooper and Blankenhorn, 1938; France, Bates, Barker and Matthews, 1938; Sydenstricker, 1938; and Rachmilewitz and Glueck, 1938). At first only a few cases were treated but later papers (Spies, Grant, Stone and McLester, 1938; and Sydenstricker, Schmidt, Fulton, New and Geeslin, 1938) reported its use in hundreds of cases. Several workers (France, Bates, Bailey and Matthews, 1938; Spies, Bean and Stone, 1938; Frontali and Ferrari, 1938; Bogart, 1938;

and Spies, Aring Gelperin and Bean, 1938) have emphasized that the most dramatic response of a pellagrin to nicotinic acid therapy is the disappearance of the acute mental symptoms.

Spies, Bean and Ashe (1939) make the following summary concerning its use:

> In cases of acute or chronic pellagra in relapse it will: (a) cause fading of the fiery red lesions of the mucous membranes and diminish the Vincent's infection associated with it, (b) in most cases, restore to normal disturbed gastrointestinal function, (c) restore to normal the mental function deranged moderately or severely in acute pellagra, (d) cause fading of the dermal erythema but not cure chronic changes of the skin. In cases of subclinical pellagra, the vague ill-defined symptoms disappear and in persons subject to recurrence of the disease the development of clinical pellagra is prevented. In both clinical and subclinical pellagra, the sense of well-being, one of the attributes of health, is restored.

Spies, Walker and Woods (1939) have shown that infants and children may also suffer from nicotinic acid deficiency in areas where pellagra is endemic. Lesions characteristic of the disease are seldom seen in infancy but frequently appear in childhood. In the absence of typical lesions the use of nicotinic acid or other active compounds offers a means of confirming a diagnosis of latent pellagra. Frontali (1938) has also shown that when children fed pellagra-producing diets are given ascorbic acid, carotene, riboflavin, aneurin, and nicotinic acid, only those getting the nicotinic acid showed immediate improvement. The cutaneous and mucous lesions healed rapidly and the nervous manifestations vanished in three weeks. Cleckley, Sydenstricker and Geeslin (1939) have reported the beneficial effect of nicotinic acid in the treatment of atypical psychotic states. Katzenellenbogen (1939) working in Palestine brought about considerable improvement in 21 out of 24 cases of stomatoglossitis characterized by soreness of the tongue and angles of the mouth and sore throat. Landor (1939), however, could not cure stomatitis with nicotinic acid but did find yeast to be active. It is quite possible that these conditions may be related to the cheilosis which Sebrell and Butler (1938a) have shown to be due to a riboflavin deficiency. Selfridge (1939) has found nicotinic acid effective in restoring degenerative processes involving the auditory nerves.

The amount of nicotinic acid needed for the treatment of pellagra varies considerably. As little as 50 mgm. may be effective while in other cases 500 to 1000 mgm. per day may be required. Part of this variation may be related to the degree of assimilation, but it is more likely related to multiple deficiencies which will be discussed later.

Since nicotinic acid has been used at rather high levels, studies on its toxicity become important. The work of both Chen, Rose and Robbins (1938) and Unna (1939) indicates the very low toxicity of nicotinic acid and its derivatives. Sodium nicotinate showed a toxicity in mice and rats only when fed at levels ranging from 4 to 7 grams per kilogram body weight. The amide was found to be somewhat more toxic than the sodium salt. Unna found that prolonged oral administration of 2 grams per kilogram daily of sodium nicotinate to rats, chickens, and dogs over periods up to 2 months failed to produce toxic symptoms. The work of Chen, as well as that reported by Elvehjem, Madden, Strong, and Woolley (1938) showed that dogs receiving 2 grams of nicotinic acid per day for several days exhibited some toxicity. However, Unna suggests that the toxicity may have been due to the acidity of the nicotinic acid since he observed no ill effects with the neutralized compound in even larger doses. In any case there seems to be the same wide range between the therapeutic dose and the toxic dose for nicotinic acid as for the other vitamins.

In addition to the above results practically all investigators have found that the administration of large amounts of nicotinic acid to human beings is generally followed by sensations of heat and tingling of the skin. This feeling is accompanied by flushing and rise in skin temperature. At the peak of the flushing Spies, Cooper, and Blankenhorn (1938) found no effect on blood pressure, temperature, or respiration. Sebrell and Butler (1938) studied the quantity of nicotinic acid necessary to produce the unpleasant reactions by dividing a group of 18 normal women into 3 groups and feeding six, 50 mgm., six, 30 mgm., and six, 10 mgm. of nicotinic acid daily in aqueous solution added to tomato juice. On the twelfth day of administration one of the subjects receiving 50 mgm. daily showed an intense flushing of the face, chest and back, which appeared in from ten to fifteen minutes after administration and disappeared after about an hour. Each daily dose produced a similar reaction for eleven successive days until the dose was given in divided portions. Four of the six women on the 50 mgm. level, only two of those on the 30 mgm. level, and none of those on the 10 mgm. level showed these symptoms. The variation among individuals is probably related to rate of adsorption since intravenous injection of 10 mgm. produces a reaction within one minute. Sebrell and Butler conclude that although the reactions are disagreeable, they persist for only a short time and cause no noticeable harm, and therefore their occurrence should not be allowed to interfere with the therapeutic use of large doses of nicotinic acid.

The activity of a number of related pyridine derivatives in black tongue was determined by Woolley, Strong, Madden, and Elvehjem (1938). The active and inactive compounds are listed in table 1. It was immediately apparent that a rather specific structure is required for anti-black tongue potency. The alpha and gamma isomers of nicotinic acid (picolinic acid and isonicotinic acid) are completely inactive. All the compounds listed in which one of the ring hydrogens had been substituted by a methyl or a carboxyl group or in which a methyl group had been added to the ring nitrogen were inactive. The replacement of the carboxyl group of nicotinic acid by a sulfonic acid group or by a cyano group or the removal of the carboxyl entirely (i.e., pyridine) led in each case to inactive compounds.

TABLE 1

Anti-black tongue activity of various pyridine derivatives

ACTIVE	INACTIVE
Nicotinic acid	Pyridine
Nicotinic acid amide	Picolinic acid
Ethyl nicotinate	Isonicotinic acid
Nicotinic acid N methyl amide	Nipecotic acid
Nicotinic acid N diethyl amide	6-Methyl nicotinic acid
β-Picoline	Trigonelline
Nicotinuric acid	1-Methyl nicotinic acid amide chloride
	Quinolinic acid
	β-Aminopyridine

It appears that in addition to the acid and its amide only those compounds possess anti-black tongue potency which are capable of oxidative or hydrolytic conversion to these substances in the body. β-picoline, which might be expected to be oxidized to nicotinic acid, showed a fair degree of activity. Nicotinuric acid was also active, which indicates that the body can hydrolyze this dipeptide. Subbarow, Dann, and Meilman (1938) reported in a preliminary note that β-amino pyridine was highly active in the treatment of black tongue. However, Strong, Madden and Elvehjem (1938) were unable to demonstrate any activity and in a later note Subbarow and Dann (1938) also found β-amino pyridine to be inactive.

It is interesting that there is a close correlation between the results obtained with dogs and those reported by Dorfman, Koser and Saunders (1938) with the dysentery bacillus. Since they found β-picoline to

be completely devoid of growth-promoting activity, the organism evidently cannot oxidize the methyl group as readily as the animal body can.

Many of the compounds found active in the dog have been used in the human. Spies, Bean and Stone (1938) found nicotinic acid, nicotinic acid amide and sodium nicotinate active, diethyl amide of nicotinic acid (coramine) somewhat active, and trigonelline inactive. Sydenstricker et al. (1938) also found the activity of coramine somewhat variable. Ammonium nicotinate and ethyl nicotinate have also been found useful. Recently Vilter and Spies (1939) reported the activity of quinolinic acid in seven patients when fed at 1,000 mgm. levels. Quinolinic acid was found to be completely inactive in the dog when given orally, and since the Vilter and Spies report, McKibbin and Elvehjem (unpublished data) have tried large doses by injection with no improvement in dogs suffering from black tongue. Perhaps the human has a greater ability to decarboxylate quinolinic acid to nicotinic acid than the dog has.

Bills, McDonald and Spies (1939) have also reported the anti-pellagric activity of pyrazine 2,3 dicarboxylic acid and pyrazine monocarboxylic acid. The activity of samples of these compounds supplied by Doctor Spies has been determined with dogs in the reviewer's laboratory. In both cases some response was obtained, but the activity was much less than with nicotinic acid. It was also interesting to observe that the best response was obtained when the dogs did not show the severe symptoms and when the dog was given the compound for the first time. Dogs that had been treated with the pyrazine compound twice in succession showed no response upon the third treatment. These results suggest that the pyrazine compounds cannot take the place of nicotinic acid in all functions but may liberate nicotinic acid from certain non-essential reactions and make it available for the more important functions.

Schmelkes (1939) has prepared thiazole-5-carboxylic acid which is isoteric with nicotinic acid, and found it to stimulate the growth of dysentery bacilli. Its order of activity was one-thousandth of that of nicotinic acid. A sample of this material submitted by Doctor Schmelkes was tested in our laboratory and found to have very slight activity in the dog.

At this point we might ask, is nicotinic acid the true antipellagra factor? The answer to this question depends upon our definition of pellagra. If pellagra is defined as the disease resulting from an insufficient intake of nicotinic acid or related compounds, then nicotinic

acid is the true antipellagra factor. Spies, Grant, Stone and McLester (1938) conclude that nicotinic acid is a vitamin and that pellagra results, at least in part, from a dietary deficiency of nicotinic acid or some closely related substance. Sebrell and Butler (1939) also suggest that in order to avoid further confusion the diagnosis of pellagra should be confined to that syndrome which responds to nicotinic acid. If pellagra includes all the symptoms usually associated with a pellagrin, then nicotinic acid is only one of a group of factors essential for the cure of pellagra. In reality this differentiation is only of academic interest and a question which arises in connection with all our synthetic vitamins. The condition generally found in the field is a multiple deficiency and it is the duty of the medical worker to clear up all the deficiencies. However, in most cases nicotinic acid is the critical deficiency and it must be provided before any significant response can be obtained. Foods carrying only fair amounts of nicotinic acid may be useless in treating severe pellagra regardless of the other factors which they may carry. The difficulties encountered in treating pellagra before nicotinic acid was available substantiate this conclusion. Similar results have been obtained in the case of dogs (Elvehjem, 1939). When a single dose of wheat germ, powdered milk, or dried grass is given to a dog suffering from black tongue, no improvement is obtained. The animal is unable to digest the food sufficiently to liberate the nicotinic acid present. Foods that are richer and can be fed in smaller quantities, such as liver, kidney, lean meat and yeast, work much better.

It would, of course, be much more logical to prevent the development of pellagra than to treat it after it is apparent, but as long as severe pellagra is encountered, the use of nicotinic acid or a related compound will probably be continued. When such a treatment is used, we must remember that after the nicotinic acid deficiency is compensated for, other deficiencies may still occur—especially those due to a lack of the other members of the vitamin B complex. Many of the workers that have used nicotinic acid have observed that the peripheral nerve involvement of endemic pellagrins is relieved only by vitamin B_1. Spies and Aring (1938) have discussed this problem in detail. Both Sebrell and Butler (1938a) and Vilter, Vilter and Spies (1939) have observed riboflavin deficiency in persons ingesting over a period of time a grossly inadequate diet similar to those consumed by pellagrins. It is also now common practice to supplement the Goldberger ration for production of black tongue in dogs with thiamin and riboflavin.

The importance of vitamin B_6 as a supplement to nicotinic acid in

the treatment of pellagrins is not so clear. For a time vitamin B_6 was confused with the antipellagra factor, but Birch, György and Harris (1935) in a comprehensive paper demonstrated that vitamin B_6 was distinct from the anti-black tongue factor (nicotinic acid) and chick antidermatitis factor (pantothenic acid). Dann (1936) showed that vitamin B_6 deficiency in a rat was etiologically different from pellagra. Then there appeared a series of papers on the isolation of crystalline vitamin B_6 (Lepkovsky, 1938; Keresztesy and Stevens, 1938; György, 1938; and Kuhn and Wendt, 1938) and with the availability of the pure vitamin it was easy to show that it had no action in uncomplicated nicotinic acid deficiency. The opinion has been rather prevalent that vitamin B_6 would not play an important rôle in conjunction with nicotinic acid because the pellagrin's diet is high in corn and corn has been considered to be high in vitamin B_6. However, recent work by Black, Frost and Elvehjem (1939) indicates that corn may be very low in vitamin B_6. Spies, Bean and Ashe (1939a) found some beneficial effect of vitamin B_6 in humans. Some of the anemia observed in dogs kept on the Goldberger diet for long periods of time (Miller and Rhoads, 1933; and Spies and Dowling, 1935) may have been related to a vitamin B_6 deficiency since Fouts, Helmer, Lepkovsky and Jukes (1937) and McKibbin, Madden, Black and Elvehjem (1939) have shown that dogs develop a microcytic anemia on diets low in this factor.

The close relationship between nicotinic acid and pantothenic acid both in chemical properties and distribution has already been referred to. To date pantothenic acid has not been available for trial in human cases. However, there are certain animal experiments which suggest the importance of pantothenic acid as well as other members of the B complex.

Dogs grow very well on a modified Goldberger diet when supplemented with thiamin, riboflavin, and nicotinic acid, but if the corn in such a diet is replaced by sucrose the dogs will grow for only a short time and then begin to lose weight. The addition to the ration of 2 per cent of liver extract renders the diet complete. McKibbin, Madden, Black and Elvehjem (1939) found that a concentrate made from liver extract containing mainly factor W and pantothenic acid would supply the necessary factors when synthetic vitamin B_6 was also added. Similar results have been obtained with rats. Helmer and Fouts (1938) found that when rats on black tongue-producing diets were given nicotinic acid they grew less than when they were given the unsupple-

mented diets. Thus nicotinic acid may be the main deficiency in pellagra, but, depending upon the amount of corn used in the diet, any one of the following vitamins may be limiting: vitamin B_1, riboflavin, vitamin B_6, pantothenic acid and factor W.

Mickelsen, Waisman and Elvehjem (1938) placed chicks on a modified Goldberger diet. They grew very poorly but none of them showed any symptoms analogous to pellagra. When this ration was supplemented with nicotinic acid at a level to supply about 1 mgm. of nicotinic acid per chick per day, slightly better growth was obtained. Further work (Waisman and Elvehjem, unpublished data) has shown that a more definite response is obtained with nicotinic acid when thiamin, riboflavin, and vitamin B_6 are supplied in the basal. The addition of a factor U concentrate gives a further growth response above that obtained with nicotinic acid.

Sydenstricker, Schmidt, Geeslin and Weaver (1939) state that from their experience no substance so far tried is as rapidly curative for all manifestations of pellagra as the Cohn fraction of liver extract for intravenous use. This would give further evidence for the value of the other factors associated with nicotinic acid in a natural substance such as liver extract. However, these workers state that it was not possible to demonstrate the presence of free nicotinic acid though nicotinic acid amide might be present in small amounts. Until more accurate chemical methods are available for the estimation of nicotinic acid it is necessary to question the absence of nicotinic acid in liver extract. The preparations tested in our laboratory on dogs have all shown considerable amounts. Evidence which will be presented later suggests that most of the biological activity of liver can be ascribed to free nicotinic acid or its derivatives.

Figures for the distribution of nicotinic acid in natural foods are still rather limited. The values published by Sebrell (1934) gave the antipellagra activity of a limited number of foods. After the recognition of nicotinic acid many attempts have been made to obtain more quantitative figures for the distribution of this vitamin. Possible methods include chemical procedures, bacterial growth methods and animal assays.

The early results of Karrer and Keller (1938) based on the color produced with 2.4 dinitrochlorobenzene were definitely too low and in a later paper they (Karrer and Keller, 1939) give higher values and point out that the previous results were too low due to incomplete extraction. Vilter, Spies and Mathews (1938) have used a method

based on the same principle for the estimation of nicotinic acid in urine. The method which appears to be most satisfactory depends upon the breakdown of the pyridine nucleus with cyanogen bromide and aniline to give a yellow colored compound which can be measured colorimetrically. Swaminathan (1938) used this method on foods; Shaw and McDonald (1938) on liver extracts and Pearson (1939) on blood. Von Euler et al. (1938) used β-naphthylamine hydrochloride in place of aniline, but according to Pearson (1939) the method is four times as sensitive when aniline is used. Bandier and Hald (1939) have used p-methyl aminophenol in place of aniline and Bandier (1939) has applied this method to biological materials with satisfactory results. Askelöf and Holmberg (1939), Ritsert (1939) and Kringstad and Naess (1939) have studied further improvements. This method has been studied rather extensively in our laboratory but completely satisfactory results have not been obtained.

The bacterial growth methods have been used for the quantitative estimation of nicotinic acid in body fluids but no extensive studies have been made on foods. As far as animal assays are concerned, both the rat and the chick assays are eliminated at least for the time being. Elvehjem, Waisman and Axelrod (1939) have described the determination of nicotinic acid dependent upon the growth response obtained with standard amounts of nicotinic acid in black tongue dogs. In table 2 are summarized some of the results which have been obtained by the different methods.

As soon as the nutritional significance of nicotinic acid was recognized, it was generally assumed that its function in the animal body must be related to coenzymes I and II. However, it was not easy to obtain direct evidence for this relationship. Both coenzymes are very important in carbohydrate metabolism and they are supposed to differ in structure only by one molecule of phosphoric acid, yet they possess remarkable specificity in relation to the dehydrogenase with which they will react. In most cases a substrate together with its dehydrogenase will react with one of the coenzymes but not with the other. The quantitative estimation of the amount of coenzyme present in tissues is based upon this specificity.

The most obvious approach to any study on the function of nicotinic acid was therefore the estimation of the coenzyme content of the tissues during nicotinic acid deficiency. Von Euler and co-workers (1938) used this approach on rats but unfortunately a specific nicotinic acid deficiency was not produced in the rats. Recently von Euler and co-

TABLE 2

Nicotinic acid content of various tissues

	Mgm. per 100 grams					
Liver, fresh:						
Pig	11.8	(2)	26.5	(10)		
Beef	17.8	(3)	9.3	(5)	25.0	(10)
Sheep	12.5	(4)	47	(10)		
Dog	7.8	(9)				
Kidney, fresh:						
Pig	6.8	(2)	15.6	(10)		
Beef	19.4	(5)	16.9	(10)		
Dog	3.8	(9)				
Muscle, fresh:						
Pig heart	5.3	(2)	8.0	(10)		
Pig	4.7	(2)	3.3	(3)	10.0	(10)
Beef	4.9	(3)	3.8	(5)	10.0	(10)
Beef heart	5.9	(2)	5.1	(10)		
Salmon (canned)	6.0	(3)				
Blood:						
Pig	0.47	(6)				
Sheep	0.83	(6)				
Liver extracts:						
Powder	9–49	(7)	270	(8)		
Liquid	6–122	(2)				
Yeasts:						
Baker's dry	25.7	(1)	12.0	(5)	50.0	(8)
Baker's moist	11.0	(3)				
Brewer's dry	44.7	(3)	59.8	(4)	30–92	(8)
Brewer's moist	10.2	(3)				
Bottom dry	35.6	(1)				
Wheat:						
Whole	5.3	(4)				
Bran	5.0	(3)				
Germ	4.2	(3)	4.0	(8)		
Corn:						
Whole	1.3	(3)				
Peanut meal:						
Raw	13.0	(8)				
Extracted	16.6	(4)				
Soy bean:						
Meal	4.8	(4)				
Skim milk:						
Powder	10.5	(4)	4.3–6.2	(8)		
Eggs:						
Yolk, dry	4	(8)				
White, dry	2.5	(8)				

(1) Bandier and Hald (1939)
(2) Bandier (1939)
(3) Kringstad and Naess (1939)
(4) Swaminathan (1938)
(5) Karrer and Keller (1939)
(6) Pearson (1939)
(7) Shaw and MacDonald (1938)
(8) McKibbin and Elvehjem (Unpublished data)
(9) Ritsert (1939)
(10) Waisman, Mickelsen and Elvehjem (Unpublished data)

workers (1939) found that the tissues from rats on diets low in the B complex tend to be lower in nicotinic acid amide and cozymase than those from normal rats. However, it remains difficult to demonstrate an uncomplicated deficiency of the pyridine nucleotides or any of their precursors in the rat.

Axelrod and Elvehjem (1939) have studied the cozymase changes in dogs and pigs on deficient diets. No changes could be detected in the blood but significant decreases were observed in the liver and muscle. The brain tissue and kidney cortex maintained a normal content under the conditions of the experiment. Kohn, Klein and Dann (1939) found similar results. In dogs with acute black tongue the concentration of coenzyme-like substances (V-factor) was 70 per cent lower in the liver and 35 per cent lower in striated muscle, compared with dogs receiving liberal amounts of nicotinic acid. The decreased coenzyme content of the liver in black tongue was accompanied by an increase of 35 per cent in oxygen consumption. The kidney coenzyme content was unchanged but its ability to oxidize lactate was decreased by 50 per cent.

Studies on the blood of normal and pellagrous patients have not given completely comparable results. Using the method of Lwoff, Kohn (1938) found no significant difference between the blood of normals and pellagrins but did find an increase even in normal patients upon administration of nicotinic acid. Vilter, Vilter and Spies (1939) however, found that the blood of normal persons supports growth of B. influenzae to a much greater extent than the blood of pellagrins. After nicotinic acid therapy, the blood of the pellagrins increased to normal growth promoting activity. In a more extensive study Kohn and Bernheim (1939) have determined the V-factor (coenzymes I and II plus possibly unknown related compounds) in the blood of 53 normal individuals and 126 hospital patients. Eighty-one per cent of the normal cases fell between 50 and 80 d.e. units (equivalent to gamma of coenzyme per one ml. of corpuscles). Only 65 per cent of the pathological cases fell in this range. Pellagrins showed values that were in the lower part of the normal range. They concluded that such determinations can be of little value in the diagnosis or prognosis of pellagra. Ballif, Lwoff, Querido and Ornstein (1939) found no reduction in the nicotinic acid amide content of the blood of 10 cases of pellagra. Axelrod, Gordon and Elvehjem (1939) found 20 to 30 micrograms of cozymase per milliliter of blood in normal individuals and the value increased to 50–60 micrograms upon the ingestion of 100 mgm. nicotinic acid per

day. When the nicotinic acid was no longer supplied the cozymase in the blood gradually returned to normal value during a period of about two weeks. Kohn and Klein (1939) have been able to establish that the human erythrocyte can synthesize V-factor from nicotinic acid in vitro as well as in vivo. Since this synthesis is accompanied by an increased ability to oxidize lactic acid, they conclude that cozymase has been synthesized.

Whether the changes observed in the liver and muscle of nicotinic acid deficient animals also occur in humans and if this decrease is sufficient to account for the gross symptoms observed cannot be answered without further information. However, it is quite possible that at least part of the rapid improvement noted both in humans and animals when nicotinic acid is administered is due to the rapid formation of cozymase when the nicotinic acid part of the molecule is made available.

Elvehjem, Waisman and Axelrod (1939) have found that if the cozymase content of fresh animal tissues is converted to the equivalent amount of nicotinic acid, it makes up an appreciable amount of the total nicotinic acid determined by animal assay. Part of the nicotinic acid must be present as coenzyme II and there is undoubtedly some free nicotinic acid and amide. More definite comparison can be made when the chemical methods are accurately standardized.

A certain amount of information has been gained from studies on the urine during nicotinic acid deficiency. Fraser, Topping and Sebrell (1938) used a method for measuring nicotinic acid or related compounds in the urine based on the growth promoting properties with Shigella paradysenteriae (Sonne). No attempt was made to estimate the nicotinic acid quantitatively. They did observe a marked increase in the growth promoting power of the urine following the administration of nicotinic acid as well as a marked decrease in the case of urine from dogs with black tongue. There was a close correlation between the biological assay of the urine and the clinical condition of the animal.

Spies, Bean and Stone (1938) report a tremendous variation in the nicotinic acid content of the urine from the same individual from time to time and from person to person. The amount excreted, however, was dependent upon the diet and the amount of nicotinic acid given and the mode of administration. A pellagrin in relapse retains more of the administered acid than does a normal person of the same size. Spies, Bean and Ashe (1939) also found an increase in the concentration of coenzyme in the urine within 24 to 48 hours after giving nicotinic acid.

Another interesting study has been the relation of nicotinic acid to pigment excretion. Beckh, Ellinger and Spies (1937) reported an increase in the amount of coproporphyrin I or III in the urine of pellagrins and a decrease following the treatment of the pellagra with yeast or liver extract. Later Spies, Gross, and Sasaki (1938) found that nicotinic acid also produces a decrease of porphyrinuria in pellagrins. They also found that the porphyrinuria associated with other diseases promptly decreased following nicotinic acid therapy. Watson (1938) found that the urinary copoporphyrin in alcoholic pellagrins was not correlated with the Beckh-Ellinger-Spies test. In a later paper Watson (1939) concludes that the color reaction observed in the urine during pellagra is due to urorosein. It occurs as a chromogen which changes to a pink or red pigment upon addition of hydrochloric acid to the urine. Another pigment soluble in chloroform may also be present which appears to be indirubin. Both of these pigments may be noted in the urine of patients not having clinical pellagra and therefore further work is necessary in order to relate the appearance of these pigments directly to nicotinic acid deficiency. Meiklejohn and Kark (1939) have also found substances in the urine of pellagrins capable of giving the urorosein reaction. They were unable to find any unusual amount of coproporphyrin in four samples of urine supplied by Doctor Spies and suggest that it would be more proper to refer to the Beckh-Ellinger-Spies test as indicating the presence of pigments capable of producing the urorosein reaction.

In summary we may emphasize two important facts which apply not only to nicotinic acid but to the vitamins in general. One is the importance of the experimental animal in extending our knowledge of important nutritional factors as well as fundamental metabolism. In the case of nicotinic acid the species of animal used has been an important factor. This does not mean that the fundamental metabolism in different species varies greatly, but rather that specific deficiency is produced more easily in one species than in another. Thus the rat was used successfully in separating the antipellagra factor from thiamin, the chick for separating it from riboflavin and the dog for establishing its relation to nicotinic acid and not to the chick antidermatitis factor. When the specific information is obtained with the animal it is readily transferred to the human.

The second is the importance of recognizing the specificity of each of the individual vitamins. Each vitamin has a very limited effect on the animal body and can be used with success only when it is lacking

in the diet. As more of the water soluble vitamins are obtained in crystalline form, it is more evident that additional factors must be recognized. The full value of one factor can only be realized when it is used in conjunction with all the essential factors. Thus nicotinic acid will cure or prevent all conditions produced by a true nicotinic acid deficiency but can be of no value in the treatment of conditions produced by a deficiency of other factors and its value in the treatment of pellagrins is much more effective when all other factors are supplied in optimum amounts.

REFERENCES

ACKERMANN, D. Ztschr. Biol. **59:** 17, 1912.

ASKELÖF, E. AND C. HOLMBERG. Svensk. Farm. Tid. **43:** 301, 1939.

AXELROD, A. E. AND C. A. ELVEHJEM. Nature **143:** 281, 1939.

AXELROD, A. E., R. J. MADDEN AND C. A. ELVEHJEM. J. Biol. Chem. **131:** 85, 1939.

AXELROD, A. E., E. S. GORDON AND C. A. ELVEHJEM. Am. J. Med. Sci. In press.

BALLIF, L., A. LWOFF, A. QUERIDO AND I. ORNSTEIN. Compt. rend. soc. biol. **131:** 903, 1939.

BANDIER, E. Biochem. J. **33:** 1130, 1939.

BANDIER, E. AND J. HALD. Biochem. J. **33:** 264, 1939.

BECKH, W., P. ELLINGER AND T. D. SPIES. Quart. J. Med. **30:** 305, 1937.

BILLS, C. E., F. G. MCDONALD AND T. D. SPIES. South. Med. J. **32:** 793, 1939.

BIRCH, T. W., P. GYÖRGY AND L. J. HARRIS. Biochem. J. **29:** 2830, 1935.

BLACK, S., D. V. FROST AND C. A. ELVEHJEM. J. Biol. Chem. **132,** 65, 1940.

BOGART, C. N. J. A. M. A. **111:** 613, 1938.

CLECKLEY, H. M., V. P. SYDENSTRICKER AND L. E. GEESLEN. J. A. M. A. **112:** 2107, 1939.

CHEN, K. K., C. L. ROSE AND E. B. ROBBINS. Proc. Soc. Exper. Biol. and Med. **38:** 241, 1938.

CHITTENDEN, R. H. AND F. P. UNDERHILL. Am. J. Physiol. **44:** 13, 1917.

DANN, W. J. J. Nutrition **11:** 451, 1936.
Science **86:** 616, 1937.

DORFMAN, A., S. A. KOSER AND F. SAUNDERS. J. Am. Chem. Soc. **60:** 2004, 1938.

ELVEHJEM, C. A. Ergebnisse der Vitamins und Hormonforschung. Leipzig, 1938.
Ann. Int. Med. **13:** 225, 1939.

ELVEHJEM, C. A. AND C. J. KOEHN, JR. J. Biol. Chem. **108:** 709, 1935.

ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG AND D. W. WOOLLEY. J. Am. Chem. Soc. **59:** 1767, 1937; J. Biol. Chem. **123:** 137, 1938.

ELVEHJEM, C. A., H. A. WAISMAN AND A. E. AXELROD. J. Nutrition **17:** Supplement 11, 1939.

FILDES, P. Brit. J. Exper. Path. **19:** 239, 1938.

FOUTS, P. J., O. M. HELMER, S. LEPKOVSKY AND T. H. JUKES. Proc. Soc. Exper. Biol. and Med. **37:** 405, 1937

FOUTS, P. J., S. LEPKOVSKY, O. M. HELMER AND T. H. JUKES. Proc. Soc. Exper. Biol. and Med. **35:** 245, 1936.
FRANCE, R., R. D. BATES, W. H. BAILEY AND E. MATTHEWS. Bull. Johns Hopkins Hosp. **63:** 46, 1938.
FRASER, H. F., N. H. TOPPING AND W. H. SEBRELL. Pub. Health Repts. **53:** 1836, 1938.
FRONTALI, G. Arch. ital. Med. sper. **2:** no. 8, 1938.
FRONTALI, G. AND G. FERRARI. Minerva Medica **2:** 133, 1938.
FROST, D. V., AND C. A. ELVEHJEM. J. Biol. Chem. **121:** 255, 1937.
FUNK, C. J. Physiol. **46:** 173, 1913.
FUNK, C. AND I. C. FUNK. J. Biol. Chem. **119:** xxxv, 1937.
GOLDBERGER, J. AND R. D. LILLIE. Pub. Health Repts. **41:** 1025, 1926.
GOLDBERGER, J. AND W. H. SEBRELL. Pub. Health Repts. **45:** 3064, 1930.
GOLDBERGER, J. AND W. F. TANNER. Pub. Health Repts. **40:** 54, 1925.
GOLDBERGER, J., C. H. WARING AND D. G. WILLETS. Pub. Health Repts. **30:** 3117, 1915.
GOLDBERGER, J. AND G. A. WHEELER. Pub. Health Repts. **30:** 3336, 1915.
Pub. Health Repts. **43:** 172, 1928.
GOLDBERGER, J., G. A. WHEELER, R. D. LILLIE AND L. M. ROGERS. Pub. Health Repts. **43:** 1385, 1928.
GUHA, B. C. Biochem. J. **25:** 945, 1931.
GYÖRGY, P. J. Am. Chem. Soc. **60:** 983, 1938.
HARRIS, L. J. Brit. Med. J. **2:** 1243, 1937.
HELMER, O. M. AND P. J. FOUTS. J. Nutrition **16:** 271, 1938.
HUBER, C. Liebig's Ann. Chem. **141:** 271, 1867.
JAHNS, E. Ber. Chem. Ges. **18:** 2518, 1885.
JUKES, T. H. J. Biol. Chem. **117:** 11, 1937.
J. Am. Chem. Soc. **61:** 975, 1939.
KARRER, P. AND H. KELLER. Helv. chim. Acta. **21:** 463, 1938.
Helv. chim. Acta. **22:** 1292, 1939.
KATZENELLENBOGEN, I. Lancet June 3: 1264, 1939.
KERESZTESY, J. C. AND J. R. STEVENS. Proc. Soc. Exper. Biol. and Med. **38:** 64, 1938.
KNIGHT, B. C. J. G. Biochem. J. **31:** 731, 1937.
KOEHN, C. J., JR. AND C. A. ELVEHJEM. J. Nutrition **11:** 67, 1936.
KOHN, H. I. Biochem. J. **32:** 2075, 1938.
KOHN, H. I. AND F. BERNHEIM. J. Clin. Investigation **18:** 585, 1939.
KOHN, H. I. AND J. R. KLEIN. J. Biol. Chem. **130:** 1, 1939.
KOHN, H. I., J. R. KLEIN AND W. J. DANN. Biochem. J. **33:** 1432, 1939.
KOSER, S. A., A. DORFMAN AND F. SAUNDERS. Proc. Soc. Exper. Biol. and Med. **38:** 311, 1938.
KRINGSTAD, H. AND T. NAESS. Ztschr. physiol. Chem. **260:** 108, 1939.
KUHN, R., P. GYÖRGY AND T. WAGNER-JAUREGG. Ber. Chem. Ges. **66:** 317, 576, 1034, 1577, 1933.
KUHN, R. AND H. VETTER. Ber. Chem. Ges. **68:** 2374, 1935.
KUHN, R. AND G. WENDT. Ber. Chem. Ges. **71:** 1118, 1938.
LANDOR, J. V. Lancet June 17: 1368, 1939.
LEPKOVSKY, S. Science **87:** 169, 1938.

LEPKOVSKY, S. AND T. H. JUKES. J. Biol. Chem. **111:** 119, 1935; **114:** 109, 117, 1936.
LORENZ, W. F. Pub. Health Repts. **31:** 221, 1916.
MCCOLLUM, E. V., N. SIMMONDS AND H. T. PARSONS. J. Biol. Chem. **33:** 411, 1919.
MCKIBBIN, J. M., R. J. MADDEN, S. BLACK AND C. A. ELVEHJEM. Am. J. Physiol. **128:** 102, 1939.
MCLESTER, J. S. Ann. Int. Med. **8:** 475, 1934.
MEIKLEJOHN, A. P. AND R. KARK. New England Med. J. **221:** 519, 1939.
MICKELSEN, O., H. A. WAISMAN AND C. A. ELVEHJEM. J. Biol. Chem. **124:** 313, 1938.
MILLER, D. K. AND C. P. RHOADS. Proc. Soc. Exper. Biol. Med. **30:** 540, 1933.
MUELLER, J. H. J. Biol. Chem. **120:** 219, 1937.
PEARSON, P. B. J. Biol. Chem. **129:** 491, 1939.
RACHMILEWITZ, M. AND H. I. GLUECK. Brit. Med. J. **2:** 346, 1938.
RAMSDELL, R. L. AND W. H. MAGNESS. Am. J. Med. Sci. **185:** 568, 1933.
RHOADS, C. P. AND D. K. MILLER. Science **81:** 159, 1935.
RITSERT, K. Klin. Wchnschr. **18:** 934, 1939.
RUFFIN, J. M., G. MARGOLIS, L. MARGOLIS, S. G. SMITH AND D. T. SMITH. J. Clin. Investigation **17:** 529, 1938.
SALMON, W. D. AND N. B. GUERRANT. Science **73:** 243, 1931.
SCHMELKES, F. C. Science **90:** 113, 1939.
SEBRELL, W. H. Pub. Health Repts. **49:** 754, 1934.
J. A. M. A. **110:** 1665, 1938.
SEBRELL, W. H. AND R. E. BUTLER. J. A. M. A. **111:** 2286, 1938.
Pub. Health Repts. **53:** 2282, 1938a.
Pub. Health Repts. **54:** 2121, 1939.
SEBRELL, W. H., D. J. HUNT AND R. H. ONSTOTT. Pub. Health Repts. **52:** 235, 1937.
SEBRELL, W. H., R. H. ONSTOTT, H. F. FRASER AND F. S. DAFT. J. Nutrition **16:** 355, 1938.
SELFRIDGE, G. Annals Otology, Rhinology and Larynology **48:** 39, 1939.
SHAW, G. E. AND C. A. MACDONALD. Quart. J. Pharm. Pharmacol. **9:** 380, 1938.
SMITH, D. T., E. L. PERSONS AND H. I. HARVEY. J. Nutrition **14:** 373, 1937.
SMITH, D. T. AND J. M. RUFFIN. Arch. Int. Med. **59:** 631, 1937.
SMITH, D. T., J. M. RUFFIN AND S. G. SMITH. J. A. M. A. **109:** 2054, 1937.
SPIES, T. D. J. A. M. A. **104:** 1377, 1935.
SPIES, T. D. AND C. P. ARING. J. A. M. A. **110:** 1081, 1938.
SPIES, T. D., C. D. ARING, J. GELPERIN AND W. B. BEAN. Am. J. Med. Sci. **196:** 461, 1938.
SPIES, T. D., W. B. BEAN AND W. F. ASHE. Arch. Int. Med. **12:** 1830, 1939.
J. A. M. A. **112:** 2414, 1939a.
SPIES, T. D., W. B. BEAN AND R. E. STONE. J. A. M. A. **111:** 584, 1938.
SPIES, T. D., A. B. CHINN AND J. B. MCLESTER. J. A. M. A. **108:** 853, 1937.
SPIES, T. D., C. COOPER AND M. A. BLANKENHORN. J. A. M. A. **110:** 622, 1938.
SPIES, T. D. AND A. S. DOWLING. Am. J. Physiol. **114:** 25, 1935.

SPIES, T. D., J. M. GRANT, R. E. STONE AND J. B. MCLESTER. South Med. J. **31:** 1231, 1938.
SPIES, T. D., E. S. GROSS AND Y. SASAKI. Proc. Soc. Exper. Biol. and Med. **38:** 178, 1938.
SPIES, T. D., A. A. WALKER AND A. W. WOODS. J. A. M. A. **113:** 1481, 1939.
STANNUS, H. S. AND C. R. GIBSON. Quart. J. Med. **27:** N. S. 3, 211, 1934.
STREET, H. R. AND G. R. COWGILL. Proc. Soc. Exper. Biol. and Med. **37:** 547, 1937.
STRONG, F. M., R. J. MADDEN AND C. A. ELVEHJEM. J. Am. Chem. Soc. **60:** 2564, 1938.
SSYMANSKA, R. M. AND C. FUNK. Chem. Zelle. u Gewebe **13:** 44, 1926.
SUBBAROW, Y. AND W. J. DANN. J. Am. Chem. Soc. **60:** 2565, 1938.
SUBBAROW, Y., W. J. DANN AND E. MEILMAN. J. Am. Chem. Soc. **60:** 54, 1938.
SUZUKI, V., T. SHIMAMURA AND S. ODAKE. Biochem. Ztschr. **43:** 89, 1912.
SWAMINATHAN, M. Ind. J. Med. Res. **26:** 427, 1938.
SYDENSTRICKER, V. P. J. Med. Assoc. Georgia **27:** 321, 1938.
SYDENSTRICKER, V. P., H. L. SCHMIDT, M. C. FULTON, J. S. NEW AND L. E. GEESLIN. South. Med. J. **31:** 1115, 1938.
SYDENSTRICKER, V. P., H. L. SCHMIDT, L. E. GEESLIN AND J. W. WEAVER. Am. J. Med. Sci. **197:** 755, 1939.
UNDERHILL, F. P. AND L. B. MENDEL. Am. J. Physiol. **83:** 589, 1928.
UNNA, K. J. Pharmacol. and Exper. Therap. **65:** 95, 1939.
VILTER, R. W. AND T. D. SPIES. Lancet Aug. 19: 423, 1939.
VILTER, S. P., T. D. SPIES AND A. P. MATHEWS. J. Biol. Chem. **125:** 85, 1938.
VILTER, R. W., S. P. VILTER AND T. D. SPIES. J. A. M. A. **112:** 420, 1939.
VOEGTLIN, C. The Harvey Lectures 87, 1919–20.
VOEGTLIN, C., H. M. NEILL AND A. HUNTER. Hyg. Lab. Bull. no. 116, 7, 1920.
VON EULER, H., H. ALBERS AND F. SCHLENK. Ztschr. physiol. Chem. **237:** 1, 1935.
VON EULER, H., H. HEIWINKEL, M. MALMBERG, I. ROBEZNIEKS AND F. SCHLENK. Ark. Kemi Mineral. Geol. 12A no. 25: 1, 1938.
VON EULER, H. AND M. MALMBERG. Biochem. Ztschr. **284:** 455, 1936.
VON EULER, H., F. SCHLENK, L. MELZER AND B. HÖGBERG. Ztschr. physiol. Chem. **258:** 212, 1939.
WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. **275:** 464, 1935.
WATSON, C. J. Proc. Soc. Exper. Biol. and Med. **39:** 514, 1938.
Proc. Soc. Exper. Biol. and Med. **41:** 591, 1939.
WHEELER, G. A., J. GOLDBERGER AND M. R. BLACKLOCK. Pub. Health Repts. **37:** 1063, 1922.
WILLIAMS, R. R. J. Biol. Chem. **29:** 495, 1917.
WOOLLEY, D. W., F. M. STRONG, R. J. MADDEN AND C. A. ELVEHJEM. J. Biol. Chem. **124:** 715, 1938.
WOOLLEY, D. W., H. A. WAISMAN, AND C. A. ELVEHJEM. J. Am. Chem. Soc. **61:** 977, 1939.

THE PHYSIOLOGY OF ARTICULAR STRUCTURES[1, 2]

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In contrast to numerous detailed descriptions of the anatomical features of joints, few comprehensive studies of their physiological properties are available. The statement made by Bichat (33) in 1799, that "no part of the physiology of the skeleton abounds more in hypothesis and less in discoveries than the synovial system," still holds a considerable element of truth. Yet today, articular disorders rank foremost among the crippling diseases. This challenge cannot be met without gaining a better insight into the physiological principles upon which the function of joints is based. Although many questions concerning the growth and activity of articular structures remain as yet unanswered, it is hoped that this presentation will facilitate a review of the available experimental evidence and stimulate further investigation. A summary of the development and anatomy of articulations will assist in correlating form and function. The physiological significance of the articular elements will be considered separately and their close interaction discussed.

ARTICULAR STRUCTURES. Articulations develop according to an inherited pattern, although their anatomy may be altered by ontogenetic influences (31, 41, 169, 214). In human embryos, the anlage of most joints is complete at the beginning of the third fetal month (157). It consists, typically, of two chondrogenous zones with an interposed remnant of mesenchyme. In the latter, liquid hyaloplasm appears (169, 213, 231, 239), probably elaborated by the mesenchymal cells (171, 269). These retract peripherally to enclose with their densely packed fibrils the future joint cavity, thus forming the embryological equivalent of the synovial membrane.

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The nature of the tissue lining joint spaces has been widely debated, but much of this discussion is of historical interest only. Suffice it to say that it has been claimed to be simple (123), stratified (236), glandular (121, 280) epithelium, single (126) or multilayered (151, 258) endothelium, fixed connective tissue (5, 33, 37, 40, 78, 110, 111, 175, 267), metaplastic cartilage (169), a reticulo-endothelial organ (88) and specialized connective tissue (176, 269). To Hueter (132) we are indebted for a fundamental description of the synovialis as a connective tissue structure, which, in essence, still holds. Microscopically, it appears as a substantial membrane of variable thickness, whose inner aspect shows a smooth, glistening surface, particularly where it overlies ligaments, tendons and menisci, while in other areas it has a dull lustre. Plicae or folds are numerous and occasional trabeculations occur, forming partially closed off compartments. An inner layer, the intima, can be lifted off easily in some parts, but not in the peripheral zone and over the tendons and menisci, where it adheres firmly. The intima may extend for a short distance onto the marginal cartilage as it does regularly in human embryos, while the outer fibrous layer forms a less complete covering, blending with the perichondrium and periosteum. Microscopically, two layers are again distinguishable, but it is readily apparent that there is no definite line of demarcation, and that the degree of differentiation is more marked in certain areas. The intimal cells near the articular surface are large, round or polygonal, with coarsely granular cytoplasm. Their nuclei are oval and their nucleoplasm shows a chromatin network. The cellular arrangement is single or multilayered, simulating endothelium or cuboidal epithelium, but there is no regularity of such patterns and a basement membrane has never been demonstrated. The synovial lining cells have protoplasmic processes which extend both parallel to the surface, where they interlace and anastomose with those of other cells, and into the deeper fibrous layer. The structure of the intima varies considerably in different regions of the membrane. Hammar (111) distinguished a cellular and a cell-poor type while Franceschini (88) contrasted a simple, acellular, poorly vascularized form closely related to the underlying fibrous stratum, with one in which he found histological characteristics of the reticulo-endothelial system. Occasionally, cells or cell groups are seen lying on the surface. More commonly, the cellular elements are separated from the cavity by collagenous material of varying thickness. Elastic fibres are scarce in the intimal layer. In the deeper fibrous stratum, the cells are smaller and have fewer, blunter and shorter

processes. The principal difference lies in the predominance of the intercellular substance. Broad bands of collagen and a slightly greater number of elastic fibres are present. Adipose tissue, of which there is a generous amount in the subintimal layer, particularly in early life (89), diminishes in the outer part of the capsule. From both the cellular and the cell-poor areas of the synovial surface, but more frequently from the former, villi of various sizes project into the joint cavity. The presence of blood vessels in the articular membrane was first demonstrated by Hunter (134) who described the "circulus articuli vasculosus". Subsequently, the vascular supply of the synovial membrane was studied histologically (37, 40, 110, 111, 176) and by arterial injection (172, 220, 259). A well-marked vascularity of the synovialis was ascertained and the capillaries were found to extend through the fibrous layer close to the synovial surface. Böhm (37) and others (164, 170, 260) failed to demonstrate lymphatic channels by intra-articular injection and disputed von Mosengeil's contention (183) that well-defined stomata connected the articular cavity with the lymphatic system. While Hueter (132) made the first definite histological observation of lymphatics in synovial tissue, Tillmanns (260) could, by direct injection of silver nitrate into the membrane and a counter-injection of the vascular tree, demonstrate a subintimal and a deeper plexus of lymphatics. Much confirmatory evidence has since been presented (21, 57, 83, 158, 172, 185, 186, 284). The finding of nervous tissue elements in the synovialis has been reported with great unanimity (65, 111, 151, 155, 192, 196, 209, 227, 247, 256). In a comprehensive study, Gerneck (95) reported the existence of a myelinated ground plexus and a superficial plexus of non-myelinated fibres. The type of endorgans indicated that both vasomotor and sensory functions are served.

Recently some workers (88, 159, 176, 228, 270) have suggested that the synovial intima represents connective tissue adapted to specific functions such as elaboration of synovial fluid constituents and reticuloendothelial activity. King (147) claimed to have demonstrated Golgi material in the cytoplasm of the synovial cells. While interesting perspectives have been disclosed in these studies, they rely largely on disputed staining methods. Other evidence is required to lift them out of the realm of speculation.

With few exceptions, the articular surfaces of bone are covered by hyaline cartilage. Grossly, this is of bluish white, ground-glass appearance. Its structural components are the typical cartilage cells housed in lacunae, an intercellular system of fibrils and the undiffer-

entiated hyaline matrix. The preponderance of matrix over cells is striking. Superficially, the cells are flat and lie parallel to the joint surface. In the subjacent, transitional layer, the cells are irregular, the lacunae larger and grouped together. The cell processes exhibit branchings and form plexuses. In a third zone, the cells are round and large, with wide, blunt processes, and arranged in rows perpendicular to the articular surface. The lowermost layer extends to the line of demarcation beyond which the matrix is calcified. Adult human cartilage is avascular (134) and has not been shown to contain lymphatics or nervous tissue (77, 111, 125, 260).

Some joints are supplied with menisci and labra. The structure of the latter resembles that of the synovial membrane, and metaplasia to fibrocartilage is common. Tendons may be intimately associated with the joint capsule, so that, at times, they take the place of the fibrous stratum. Bursae are frequently connected with the joint cavity proper, or located as separate structures between tendons and bony prominences. Their embryological derivation from a mesenchymal cleft and the histological character of the bursal wall suggest a close relationship with synovial tissue (140).

In summary, then, it may be stated that a typical diarthrosis consists of the opposing articular cartilages, the capsule connecting them and a variable amount of synovial fluid. Embryologically and histologically, the articular components are of mesenchymal character. The membrane conforms in structure to connective tissue. It possesses a collagenous matrix, a rich supply of blood vessels, lymphatics and nerves, few elastic fibres and characteristic connective tissue cells. The cellular elements are sparse in the external layer, but near the lumen cell aggregates occur, representing a modification of fixed connective tissue cells. Direct communications between the lymphatics of the membrane and the articular cavity have not been demonstrated. On the other hand, no apparent barrier exists separating the articular cavity from the intercellular spaces of the synovialis. The evidence presented indicates that the joint is a tissue space rather than a body cavity. The designation of synovial tissue as a membrane, although in common use, is anatomically incorrect and should be employed with this understanding.

ARTICULAR FUNCTION. Our knowledge of the structure of joints, although far from complete, exceeds considerably that of joint function because the interest has only in recent years shifted from morphological to physiological research.

Earlier workers were concerned with the forces which hold joints together. The Webers (276) claimed to have proven that it is the atmospheric pressure, rather than the action of muscles, tendons and ligaments, which saves man from dislocating his femur in the act of walking. Later investigators (94, 234), however, showed that barometric pressure could support merely a fragment of femoral head (35 grams) in the acetabulum and argued correctly that a partial vacuum in a joint cavity, sufficient to carry the weight of an extremity (10–13 kgm.), would be a physiological curio. Allen (7) emphasized the importance of the capsular ligaments, while Fick (78) again stressed the effectiveness of atmospheric pressure. Bordier (39) suggested that the molecular cohesive force of a synovial fluid film aids in maintaining apposition of joint surfaces. This factor, of course, cannot be fully active in extremes of motion, and therefore it is evident that the supportive structures, mainly the ligaments, are in a large measure responsible for the stability of joints.

Cartilage. Articular cartilage is the final recipient of all jolts and blows exerted upon the skeleton. The physical properties by which this tissue attains its efficient buffer action have been investigated in vitro (17, 28, 99, 210) and resilience has been found to be a pre-eminent characteristic. Cartilage is almost completely elastic to frequent intermittent pressures, while continuous compression of the same total strength decreases its expansile power and lengthens the period of recovery. Much of the elastic latitude is lost by drying but returns if the desiccated material is suspended in physiological saline, even after an interval of several months. Benninghof (28) therefore considered that the mechanism of elastic action consists of exudation and reabsorption of fluid. These observations gain further significance when we consider the nutrition of this avascular tissue. In accord with Toynbee (264) and Hunter (134), some workers (45, 283) still attach primary importance to subchondral, capsular or perichondrial blood sources. Others (16, 82, 137, 194) hold that such blood supply serves as a source of nutrition only for the immediately adjacent cartilaginous parts. The prevailing opinion (9, 36, 138, 154, 169, 254) is that the bulk of articular cartilage derives its nourishment from the synovial fluid. Evidence for this has been gained from the study of spontaneous and experimentally created loose bodies in joints. While those consisting of bone undergo necrosis, cartilaginous fragments remain viable for many weeks (26, 114). No conclusive data have been presented elucidating the mechanism by which nutritive material enters and

waste leaves the cartilaginous substance, but it is assumed that metabolites pass along the intercellular system of fibrils (169). Müller (187) conceived of the exchange as a physical process, induced by alternating pressure and suction. Other workers (116, 182) searched for a clue by studying, in vitro, the permeability of cartilage to chemical radicals. Electrolytes supposedly penetrated poorly and without selectivity (116). Hydrogen and hydroxyl ions exhibited even less tendency to diffuse. Nonelectrolytes entered in indirect relation to their molecular size and in direct variance with lipoid solubility. In these experiments the presence of the diffusing substances in cartilage itself was not considered, a factor which would influence the establishment of equilibria.

More definite data have been obtained from studies pertaining to metabolism of cartilage. Its respiration is nearly nil (47, 49, 160) and, like muscle, it possesses the faculty of anaerobic oxidation. The metabolic rate is one-tenth that of connective tissue but, per cell, is of the same order as that of other tissues. Kuwabara (160), confirmed by Bywaters (49), demonstrated the presence of a dehydrogenase (hexose-phosphate). Interesting relationships have been revealed between glycogen content, glycolysis and growth of cartilage. Hoffman et al. (127) demonstrated that epiphyseal cartilage of cattle embryos contains nearly 3.5 times as much glycogen as adult tissue or, as expressed by Gendre (93), the glycogen content varies with the state of hypertrophy. Bywaters found the metabolism of fetal epiphyseal rabbit cartilage ten times that of adult cartilage, or double the amount per cell. The higher glycogen content of fetal epiphyseal cartilage could also be demonstrated histologically in specimens stained with Best's carmine (117, 136). In older animals, glycogen was occasionally absent in epiphyseal cartilage. The glycogen content of the epiphyses is smaller and more labile than that of rib cartilage, a finding which would agree with Dickens' (70) report of higher metabolic rates in the latter. In addition to glycogen, articular cartilage is known to contain lactic acid, collagen, chondroitin sulfuric acid and calcium salts. Its exact chemical composition has not been determined with any degree of completeness.

Growth and repair of cartilage are more fully understood. In a carefully controlled histological study on epiphyseal cartilage of mice and rats, Elliott (74) was able to show that mitosis is the normal mechanism of cell division in immature cartilage. As maturity progresses, amitosis prevails and in adult tissue it is the sole form of growth. The area of maximal activity appears to be located a short distance beneath the articular surface. Bennett and Bauer (23) likewise concluded that

following experimental injury, proliferative activity is greater in the deeper zones. Recent experimental work (248, 249, 250) if confirmed will aid in evaluating the rôle played by endocrine factors in the growth of cartilage. Anterior pituitary extract was found to induce hypertrophy and hyperplasia in the transitional zone, followed by liquefaction and ulceration of the articular surfaces, in young guinea pigs, whereas in adult animals the degenerative changes predominated. Similar sequelae were observed after gonadectomy in immature subjects. Oral administration of thyroid extract for from two to thirty days, enhanced growth and differentiation of euhyaline cartilage in normal young guinea pigs. The indispensability for the growth of cartilage of such functional stimuli as pressure and attrition has been repeatedly postulated (29, 40, 77, 187, 225). Bennett, Bauer and Maddock (26) demonstrated that apposition of articular surfaces is required for the integrity of joint cartilage and that proliferative reactions, following experimental trauma, are stronger in weight-bearing than in passive areas. Reyher (214) found that when he immobilized the extremities of animals for sixty-two days, only the non-contiguous joint surfaces showed evidence of degeneration. Proper function, of course, precludes a multitude of pathological states not within the scope of this review. However, the deleterious effect of continuous pressure on joint cartilage as revealed in clinical experiences (58, 153) and experimental studies (157, 224) should be mentioned because it tends to confirm Müller's concept of the nutrition of cartilage. Histological studies have shown that, with advancing age, the superficial zone undergoes fibrillary degeneration, while the vertical stratum moves toward the surface (10, 11, 24, 25). Cataplastic changes were found to begin almost immediately upon cessation of growth.

From the reaction of cartilage to injury, further information concerning its growth has been obtained. Leidy (1849) (167) was the first to observe that experimental cartilaginous fractures did not unite. Redfern (211), two years later, added that repair of cartilaginous wounds may take place by invading fibrous tissue. A host of experimental studies have since confirmed these results, with slight modifications. A few workers found that injuries confined to cartilage showed no evidence whatever of regeneration (96, 107, 108). Somewhat more numerous and, in general, of more recent origin are observations of slight or limited healing in such wounds (15, 56, 202). Häbler (109) reported that superficial cuts remained essentially unchanged for three hundred and four days and that only in one of sixteen specimens was there any microscopic evidence of proliferation. Bennett, Bauer and Maddock

(26) published an experimental study, controlled as to location of injury and age of animals. They observed that experimental lesions in normal young adult dogs showed only slight proliferation after as long as twenty-eight weeks, when the injury did not involve subchondral bone or perichondrium and was located on weight-bearing surfaces. Ultimately, some of these defects were filled in with tissue having an imperfect resemblance to hyaline cartilage. On passive surfaces, no repair at all was observed in two joints after four and twelve weeks respectively. Bennett and Bauer (23) demonstrated that the proliferative ability of joint cartilage in immature animals does not exceed that of grown subjects. Most observers agree that wounds in cartilage extending into the subchondral bone, or located close to the perichondrium or the synovial membrane, fill in rapidly with a fibrous pannus. This, in turn, may undergo metaplasia to fibrocartilage and at times to imperfect hyaline cartilage. Seggel (243), Fasoli (76) and Shands (245) occupy a somewhat different position, emphasizing more strongly the regenerative power of cartilage.

Cartilage thus has been shown to be an elastic, avascular tissue with a low ratio of cells to matrix. Its chief, if not sole, source of nourishment is the synovial fluid. The mechanism by which nutritive material gains entrance has not been established. The low oxygen requirement and limited reparative ability confirm the fact that cartilage is a relatively inactive tissue.

Synovial membrane. The mechanical properties of the articular capsule are determined by its fibrous layer, rather than by the intima. As might be expected from its anatomical structure, the synovial membrane has little elasticity. If fresh capsular tissue is tested in vitro, the elastic resistance is about thirty times that of a sheet of rubber of equal thickness (66). Elastic expansion can therefore account for only a small increase in surface area. Since, in the physiological state, a large portion of the cavity walls is collapsed by the pull of tendons, additional space may be made available through muscular relaxation. Direct determinations of the potential joint volume by instillation of fluids are subject to many errors. The results to date show such wide discrepancies as 60 and 300 cc. for the knee joint of a normal adult male (77, 201). When there are sudden, large accumulations of fluid, actual stretching of the membrane may take place. But like tendinous tissue, it possesses a high resistance to tear (97 kgm. per sq. cm.). The marked pliability, considered by Danckelmann (66) the outstanding physical characteristic, aids in withstanding the severe stresses of joint motion.

The metabolism of the synovial membrane, per cell, was found to be

of the same magnitude as that of other tissues (47). Respiratory quotients varying in the majority between 0.71 and 0.72 suggest predominant oxidation of fat. A positive methylene blue reaction indicated the presence of dehydrogenase.

In contrast to cartilage, the articular membrane has a pronounced and undisputed regenerative ability (142, 242, 282). Key (142), in a comprehensive study, observed a series of hemisynovectomized knee joints of rabbits up to one hundred and four days. After a primary polymorphonuclear reaction in the fibrin clot, clasmatocytes and monocytes appeared and organization by connective tissue was usually complete within six days. A layer of collagen formed on the repaired surface, beneath which fibroblasts were seen in aggregates. On the tenth day, the number of cells in this layer had increased markedly. In the ensuing stage, a diminution of cellular activity paralleled by abundant deposition of collagen was noted. Villi developed and after about sixty days, a nearly normal synovial membrane was present. Restitution had thus taken place from the external or deeper tissues, with little or no growth from the edges. From these and other experimental studies, Key (142, 144) concluded that joint cavities are clefts lined by slightly modified connective tissue cells. Wolcott (282) reported that following typical synovectomy, the excised membrane regenerated completely, both in adult and in young dogs. Thirty to one hundred days postoperatively, the new tissue was nearly indistinguishable from a normal articular capsule in structure, size and contour.

Synovial fluid. Cytology. Normal joints contain varying amounts of synovial fluid. Rarely can one aspirate more than a drop from the knee or shoulder joints of the smaller laboratory animals (rabbit, cat and dog) (216, 275). In young western cattle from 3 to 7 cc. of synovial fluid can be obtained from the carpometacarpal joint, whereas 15 to 40 cc. can be aspirated from the astragalotibial joint (19). The average quantity obtainable from a normal human knee joint is 0.45 cc. with a minimal-maximal variation of 0.13 to 2.0 cc. (60).

The earliest reports (111, 161) indicated that normal synovial fluid is relatively cell-poor but gave little information as to the percentage of each cell type. Subsequent studies (19, 144, 275) agreed as to its cell content but revealed marked variations in the total number of cells and the percentage number of any one type in the various species of animals. Key (144) working with rabbits found that shoulder joint fluid contained 175 to 225 cells per cu.mm. and approximately the same number of erythrocytes. By means of supravital staining, he

established the following differential nucleated cell count: monocytes, 58 per cent; clasmatocytes, 15 per cent; indeterminate phagocytes, 14 per cent; leukocytes, 5 per cent; primitive cells (resembling small lymphocytes), 1 per cent, and synovial cells, 3 per cent. Warren, Bennett and Bauer (275) found somewhat higher total nucleated cell counts in fluid from the knee joints of normal rabbits but the average differential cell count was approximately the same. These same workers (220, 275) reported average nucleated cell counts of 112, 131 and 182 cells per cu.mm. in three large series of astragalotibial joint fluids and total counts of 213 and 222 cells per cu.mm. in two series of carpometacarpal fluids (cattle). The average nucleated cell count for astragalotibial fluid was: monocytes, 36.4 per cent; clasmatocytes, 15 per cent; unclassified phagocytes, 3.9 per cent; lymphocytes, 40.1 per cent; polymorphonuclear leukocytes, 2.2 per cent; synovial cells, 1.2 per cent, and unclassified cells, 1.2 per cent. The differential cell counts on the more cellular carpometacarpal fluid averaged: monocytes, 63 per cent; clasmatocytes, 7.2 per cent; unclassified phagocytes, 3 per cent; lymphocytes, 23 per cent; polymorphonuclear leukocytes, 1.2 per cent; synovial cells, 1.7 per cent, and unclassified cells, 1 per cent. It was thought that cellular debris associated with the regularly occurring cartilage defect in the carpometacarpal joints was sufficient stimulus to cause the increase in the total number of nucleated cells and the higher percentage of mononuclear phagocytes. The highest average nucleated cell count (963 per cu.mm.) was observed in the synovial fluid specimens obtained from dogs (275). The average differential nucleated cell count of the dog fluids was very similar to that of the carpometacarpal fluids of cattle. Erythrocytes were absent in many of the synovial fluid specimens obtained from the various species of animals (19, 275), thus indicating that they are not normal constituents of synovial fluid. When found, they are undoubtedly the result of trauma incident to the aspiration. Neither the total number of nucleated cells nor the individual cell types are influenced by variations of the blood cytology (19). Post mortem migration of cells into the joint may take place but occurs without any appreciable alteration in the percentage of individual cell types (275).

Knowledge of normal human synovial fluid cytology was very meagre until recently because no one of the previous workers (86, 111, 144, 148, 161, 177) had examined more than one or two fluids. A study of twenty-nine fluids obtained postmortem from human knee joints showing no evidence of disease has been made in our laboratory (60). The

total number of nucleated cells varied from 13 to 180 cells per cu. mm., the average being 63 cells per cu.mm. The total cell counts on knee joint fluids from the same individual in most instances were approximately the same. Again it was found that the synovial fluid cytology was never a reflection of the blood cytology. Failure to detect erythrocytes in eight of these fluids serves as further evidence that they are not normally present. The average differential nucleated cell count was: monocytes, 47.9 per cent; clasmatocytes, 10.1 per cent; unclassified phagocytes, 4.9 per cent; lymphocytes, 24.6 per cent; polymorphonuclear leukocytes, 6.5 per cent; synovial cells, 4.3 per cent, and unclassified cells, 2.2 per cent. Eosinophils and basophils were not observed in any one of the twenty-nine synovial fluids examined, indicating that they are rarely present in normal synovial fluid.

The average total cell count of human synovial fluid is lower than that of any animal species thus far studied although marked individual variations were noted. The total cell count in many instances might have been higher if the joints had been subjected to normal use up to the time of death. Again mononuclear phagocytes represented the predominant cell type. From previous studies (19, 275) it would appear that their chief function is the removal of wear and tear products (debris and particulate matter) resulting from minor traumata and insults. That type and degree of trauma influence synovial fluid cytology can readily be demonstrated. Injection of normal saline into a rabbit's knee joint (19) results in an immediate rise in the polymorphonuclear leukocytes which are replaced subsequently by mononuclear phagocytes. Apparently such mild irritation produces sufficient change in the subsynovial capillaries to allow for an immediate intra-articular migration of polymorphonuclear leukocytes, whereas the mononuclear phagocytes arising from the synovial tissue and tissue fluids are much slower to respond (146). Therefore, it seems reasonable to assume that the variations in total number of cells and individual cell types encountered in normal synovial fluid probably represent the intra-articular response to the inconstant trauma incident to daily use. It is of further interest that the highest cell counts are observed in those joints showing the greatest wear and tear changes in consequence of increasing age and long-continued use (24, 25).

Since synovial fluid serves chiefly as a lubricant for the articular surfaces and as a source of nourishment for articular cartilage, it is not surprising that it contains relatively few cells. If the joint cavity is an enlarged tissue space (62, 132, 144, 147), lined by modified connective

tissue, the cytology of synovial fluid may be considered representative of that of tissue fluid.

Physical Characteristics. Complete physical and chemical characterization of normal synovial fluid has been possible in cattle (220), and normal human fluid has been compared in enough cases to indicate that the fluids are essentially alike (222).

Normal synovial fluid is a clear, pale yellow, viscous liquid. Its pressure has not been adequately studied. No satisfactory method which takes into account variations due to motion and muscle tone has as yet been devised. In a moderately well-controlled study, Müller (188) examined the synovial fluid pressure on fresh cadavers, anesthetized humans, anesthetized dogs and on non-anesthetized standing dogs. He concluded that a slightly negative pressure exists in normal joints, ranging from -2 to -12 cm. of water and varying with muscle tone and position.

The average specific gravity of cattle synovia is 1.010 with a maximum of 1.012 and a minimum of 1.009. In human fluids obtained post mortem from joints in which no histological changes were found in the membrane, Horiye (129) found similar figures ranging from 1.008 to 1.015.

The average content of total solids of cattle fluid is 2.08 grams per 100 grams with a maximum of 3.89 and a minimum of 1.67, as compared with an average of 8.73 in the serum. These figures correspond closely with those of Horiye (129) for post mortem human fluids (1.20 to 3.93 per cent), but are slightly lower than the value given by Fisher (83) for normal human fluids obtained post mortem (4.4 per cent).

The average freezing point of cattle fluid is $-0.535°$ with a maximum of $-0.556°$ and a minimum of $-0.590°$, as compared with an average for the serum of $-0.590°$.

The relative viscosity of normal synovial fluid varies markedly in different joints. In fluid from the astragalotibial joints of normal cattle, the average is 3.7 at 25°C., with variations from 2.8 to 4.2. Fluid from the carpometacarpal joints, on the other hand, has an average viscosity of 64, with variations from 29 to 129. Normal human fluid has been found to have an even higher viscosity, with an average of 124 and a range from 51 to 209 (222). Schneider (237) reported variations from 3.9 to the unlikely value of 1490 in fluids obtained post mortem from patients without joint disease. Kling (149) found viscosities of 10.7 and 20 in two normal numan fluids. The factor responsible for the high viscosity appears to be mucin. Following precipitation of the

mucin, the viscosity of the fluid approaches that of water. The cause of the marked variations in different joints is not known. They correspond, in general, with the variations in mucin content and, furthermore, seem to be associated with the degree of polymerization of the mucin.

The average osmotic pressure against Ringer-Locke solution is 365 mm. of water for the serum and 150 mm. of water for cattle fluid. By

TABLE 1

Chemical composition of normal cattle synovial fluid and serum

	PROTEIN (NOT INCLUDING MUCIN)		ALBUMIN		A/G RATIO		MUCIN	SUGAR		NON-PROTEIN NITROGEN		UREA		URIC ACID		pH	
	Se	Fl	Se	Fl	Se	Fl		Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl
	grams	*grams*	*grams*	*grams*			*grams*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		
Average*	7.87	0.886	3.97	0.712	1.10	3.90	0.138	91	66	24	21	8.5	8.2	1.84	1.55	7.42	7.31
Maximal	8.75	1.410	4.14	0.889	1.27	5.82	0.252	176	93	31	28	12.8	14.1	2.26	2.08	7.55	7.43
Minimal	7.11	0.435	3.64	0.493	0.98	2.48	0.033	58	45	18	15	6.6	6.1	1.54	1.20	7.31	7.17
Number‡	37	36	7	6	7	6	11	12	12	32	30	15	15	7	7	21	23

	CHLORIDE		BICARBONATE		PHOSPHORUS		SULPHATE		LACTIC ACID		TOTAL BASE		SODIUM		POTASSIUM		CALCIUM		MAGNESIUM	
	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl
	m. eq.	*m. eq.*	*m. eq.*	*m. eq.*	*mM*	*mM*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*
Average†	109.8	110.5	26.8	28.5	2.2	2.2	5.56	4.96	5.47	3.21	165.7	163.2	156.1	145.0	5.37	4.04	5.6	3.8	1.75	1.44
Maximal	117.1	116.3	29.5	31.8	3.0	3.0	6.03	5.42	9.71	3.80	180.6	180.8	167.9	147.8	5.87	4.40	6.2	4.3	2.22	1.72
Minimal	103.6	104.9	23.8	25.3	1.4	1.5	5.28	4.53	4.14	2.06	154.2	152.5	148.7	140.1	4.44	3.60	4.6	2.7	1.54	1.33
Number‡	31	31	13	15	15	15	6	6	5	6	18	18	6	6	6	6	15	15	8	8

* All values represent the concentration in 100 grams of water.

† All values represent the concentration in 1000 grams of water.

‡ Represents the number of fluids from which the average figures were obtained.

direct determination the average osmotic pressure difference between the fluid and the serum is 250 mm. of water. It is of interest to compare these values with the colloidal osmotic pressures calculated from the average albumin and globulin figures. Using the factors determined by Govaerts (103) for the pressure exerted per gram by serum albumin (75.4) and serum globulin (19.5), the osmotic pressure of the serum is 384 mm. of water. This agrees fairly well with the observed value of 365 mm. In the case of the fluid, however, the pressure calculated from the albumin and globulin content is only 57 mm. of water, in contrast

to the observed value of 150 mm. Mucin is the only other colloidal substance known to be present in the fluid. The osmotic pressure exerted by mucin has not been determined. If one assumes that the difference between the observed and the calculated osmotic pressures of the fluid is due to mucin, its osmotic pressure effect per gram is nine times as great as that of albumin (675 as compared with 75).

Protein Constituents. The average concentration of protein not including the mucoprotein, is 0.88 gram per 100 cc. in cattle fluid, in contrast to 7.40 grams per 100 cc. in the serum. This figure is in the same range as that found for normal human synovial fluid. In specimens obtained post mortem from patients who had had no joint symptoms and no edema, it was 1.36 grams per 100 cc., with variations from 0.23 to 2.13 grams (222). Fisher (83) reported a protein content of 1.6 per cent in normal human fluid and 0.92 per cent in cattle. Horiye (129) found variations from 0.45 to 3.15 per cent in fluids obtained post mortem from joints in which he found no histological changes in the membrane. Judging from the findings in a large number of normal and pathological fluids, we would conclude that the value of 3.15 per cent represents an abnormal fluid. Cajori and Pemberton (52), in synovial fluid from a patient with generalized edema, found a protein concentration of 1.39 per cent. The protein content reported for other body fluids is somewhat higher. Heim (122) found variations from 1.38 to 4.57 per cent in lymph, while Arnold and Mendel (13) obtained a value of 3.56 per cent. The total protein concentrations determined by Gilligan, Volk and Blumgart (98) varied from 0.25 gram per 100 grams of water in subcutaneous edema fluid to 4.36 grams in a case of ascites secondary to carcinoma. Their average value for all fluid proteins (chest, ascitic and edema fluids) was 1.49 grams per 100 grams of water.

The globulin fraction in cattle fluid, as determined by the method of Butler and Montgomery (46), averages 0.18 gram per 100 grams of water, with an average albumin content of 0.71 gram. The average albumin-globulin ratio of the fluid is 3.9, in contrast to an average ratio of 1.1 in the serum. The globulin concentration and the albumin-globulin ratio vary more in the fluid than in the serum, both in normal (222) and pathological states (52, 221). Similarly, marked variations in the albumin-globulin ratio in other body fluids were found by Gilligan, Volk and Blumgart (98). They may be due to difficulties in fractionation of the protein constituents when the total protein content is low, but more likely represent variations in capillary permeability.

The presence of serum proteins in lymph and other body fluids has

never been explained adequately. The majority of investigators (55, 63, 72, 79, 156, 274) have concluded that it is the result of slight capillary permeability to protein and subsequent concentration. Other workers (162, 163, 174, 257) consider that capillary permeability to proteins is negligible. Formation of the proteins in situ is another possibility but has never been investigated. The available evidence indicates that normal capillary walls are slightly permeable to proteins, but whether sufficiently so to explain the concentration of protein in body fluids is unknown.

The low globulin concentration and high albumin-globulin ratio of the fluid compared to those of the serum are in accord with the results obtained for other body fluids (lymph, edema fluid and ascitic fluid) (73, 80, 100, 277, 279). If one assumes that the serum proteins enter the fluids through the capillary walls, the high albumin-globulin ratio indicates greater permeability to albumin than to globulin, corresponding with the difference in molecular weights of albumin and globulin. A similar difference is found in their rates of removal from joints (20), which will be discussed below.

In addition to albumin and globulin, synovial fluid contains mucin. It is this mucoprotein that is responsible for the high viscosity and presumably for the lubricating value and high colloidal osmotic pressure of the fluid. The concentration of mucin varies markedly in different joints. The average concentration in the astragalotibial joints of cattle is 0.14 gram per 100 cc., and in the carpometacarpal joints, 0.60 gram. Fisher (83) found 1.95 per cent mucin in normal human fluid and only 0.13 per cent in fluid from oxen. Cajori and Pemberton (52) reported a mucin content of 0.42 per cent in fluid from a patient with generalized edema. Achard and Piettre (1) found 5.7 per cent mucin in normal human fluids. We cannot account for the latter value since the maximal total protein concentration of normal fluid, including mucin, is 3.23 (222). The average mucin concentration in normal human fluids obtained in this laboratory is 0.85 gram per 100 cc., with variations from 0.55 to 1.10 grams.

The exact chemical composition of synovial fluid mucin has not been established. It has been generally accepted that it is a glycoprotein, and not a nucleoprotein as originally suggested by Hammarsten (112). Various elementary analyses have been reported (3, 128, 219, 230). Of greater importance than such analyses is the determination of the structural composition and the nature of the component protein and polysaccharide. The identity of the protein has not been

established but it probably is not a single globulin, as suggested by Meyer, Smyth and Dawson (181). The polysaccharide contains equimolar parts of acetylglucosamine and a hexuronic acid (181). Meyer, Smyth and Dawson (181) have concluded that synovial fluid polysaccharide is identical with the polysaccharides of vitreous humor, umbilical cord and group A hemolytic streptococcus. The results of enzymatic studies in our laboratory also suggest the identity of the mucins and polysaccharides of synovial fluid, vitreous humor and umbilical cord, but proof will not be possible until the nature of the proteins and polysaccharides has been established with certainty. It is significant that mucin obtained from subcutaneous tissue is also similar, physically and enzymatically, to the above mucins (218). While the majority of investigators have considered the mucin of joint fluid to be a glycoprotein, Meyer, Smyth and Dawson (181) conclude that the polysaccharide acid occurs in the fluid either free or united to protein in salt linkage only. Such an hypothesis is not in accord, however, with all of the physical and chemical properties of synovial fluid mucin.

Normal synovial fluid contains no fibrinogen as suggested by its failure to clot. The absence of fibrinogen has been corroborated in this laboratory by precipitation experiments with 1.1 M phosphate solutions at pH 6.5.

Distribution of Non-electrolytes. The average concentration of urea in cattle fluid per 100 grams of water (8.2 mgm.) is approximately the same as that of the serum (8.5 mgm.), as would be expected if serum and fluid were separated by a membrane permeable to this substance. The distribution ratios of total non-protein nitrogen (0.87) and uric acid (0.84) between fluid and serum in cattle are somewhat lower than that of urea, but of the same general magnitude. These findings are in accord with those reported by Hare and Cohen (115) for normal horse synovial fluid, and by other workers for pathological fluids (8, 51, 52, 62, 191, 221). The distribution ratio for nonprotein nitrogen in normal human fluid is 0.91 (222).

Although the average distribution ratios for urea, uric acid and nonprotein nitrogen are slightly below 1.00, in individual cases equal concentrations in fluid and serum have been observed. It is evident, therefore, that these non-electrolytes are completely diffusible through the membrane separating serum and fluid.

The average concentration of sugar in cattle fluid is, on the other hand, considerably lower in the fluid than in the serum, and the in-

dividual variations are much greater than those for other substances. These differences may be due in part to the fact that the animals were not fasting. Another explanation may be that the ante mortem struggle caused a sudden rise in blood sugar which was not reflected in the joint fluid by the time of death. Analysis of human fluids (222) shows that one cannot ascribe these variations to the presence of a non-diffusible portion of glucose in the serum as suggested by Brull (43). In most cases, the distribution ratios of sugar between fluid and serum are approximately 1.00. Complete diffusion of reducing substances has been found also in studies on plasma and glomerular urine (273).

Cholesterol and fatty acids are not found in normal fluid. This is in accord with the generally accepted theory that the capillary membrane under normal conditions is not permeable to these substances.

In summary, the distribution of non-electrolytes is consistent with that found in a dialysate of blood plasma.

Enzymes. The enzymes of normal synovial fluid have not been studied in detail. Podkaminsky (206) found that diastase, lipase and protease are present, but did not detect any catalase. The fluid of cattle has been found to have a higher average phosphatase activity than the serum. Greater variations are encountered in the fluids than in the sera. Further studies are needed in order to explain such variations and to determine the rôle of enzymatic activity in the metabolism of articular structures.

Hydrogen Ion Concentration. The average pH of cattle fluid is 7.31 as compared with an average pH of 7.42 for the serum. Few reports of the pH of normal human synovial fluid have been made. Horiye (129) found post mortem fluid to be weakly alkaline to litmus. Seeliger (241) reported its pH as 8.2 to 8.4. Boots and Cullen (38) found a pH of 7.34 in fluid from a patient with generalized edema. Pescatori (203) studied forty post mortem fluids and found an average pH of 7.95 with variations from 7.53 to 8.02. The average pH of normal human fluids obtained post mortem, as determined in this laboratory, is 7.40.

Distribution of Electrolytes. The concentrations of chloride and bicarbonate are higher in the fluid than in the serum of cattle, while the concentrations of sodium, potassium, calcium and magnesium are lower in the fluid than in the serum. The concentration of total inorganic phosphate is practically the same in fluid and serum. These distributions are, in general, such as would be expected from the laws regulating membrane equilibrium. The close agreement of the ratios

with those for edema and ascitic fluids, lymph and the "in vivo dialysate," and with the theoretical Donnan distribution ratio is apparent from table 2. The acid-base equilibrium between serum and fluid is presented graphically (see fig. 1).

The excess of chloride in the fluid bears about the same relation to the excess of protein in the serum as has been found when lymph,

TABLE 2

Comparison of the distribution ratios for synovial fluid (220), edema fluid (98, 101, 120, 168), the "in vivo dialysate" (106), ascitic fluid (67, 105, 120, 190) and lymph (13, 122) and the theoretical Donnan ratios

	SYNOVIAL FLUID	EDEMA FLUID	"IN VIVO DIALYSATE"	ASCITIC FLUID	LYMPH
$\frac{Cl_s}{Cl_f}$	0.99	0.97	0.98	0.98	0.95
$\frac{HCO_{3s}}{HCO_{3f}}$	0.94	0.96	0.97	0.97	
$\frac{PO_{4s}}{PO_{4f}}$	1.00	1.03	1.17	1.12	1.07
$\frac{Na_f}{Na_s}$	0.93	0.97	0.91	0.94	
$\frac{K_f}{K_s}$	0.75	0.73	0.78	0.94	
$\sqrt{\frac{Ca_f}{Ca_s}}$	0.83	0.80	0.76	0.84	
$\sqrt{\frac{Mg_f}{Mg_s}}$	0.88		0.66	0.86	
Theoretical Donnan ratio	0.93	0.96	0.93	0.97	

edema fluids and the "in vivo dialysate" are compared with serum. The excess of chloride in the fluid in proportion to the excess of protein in the serum, however, is slightly lower than that found for the other fluids (97). This difference may be related to the nature and relative concentration of the proteins in synovial fluid. The high-albumin-globulin ratio in the fluid would tend to increase the base-binding

power per gram of total protein. The presence of mucin may further augment the base-binding power, as indicated by the results of solubility experiments on mucin (265).

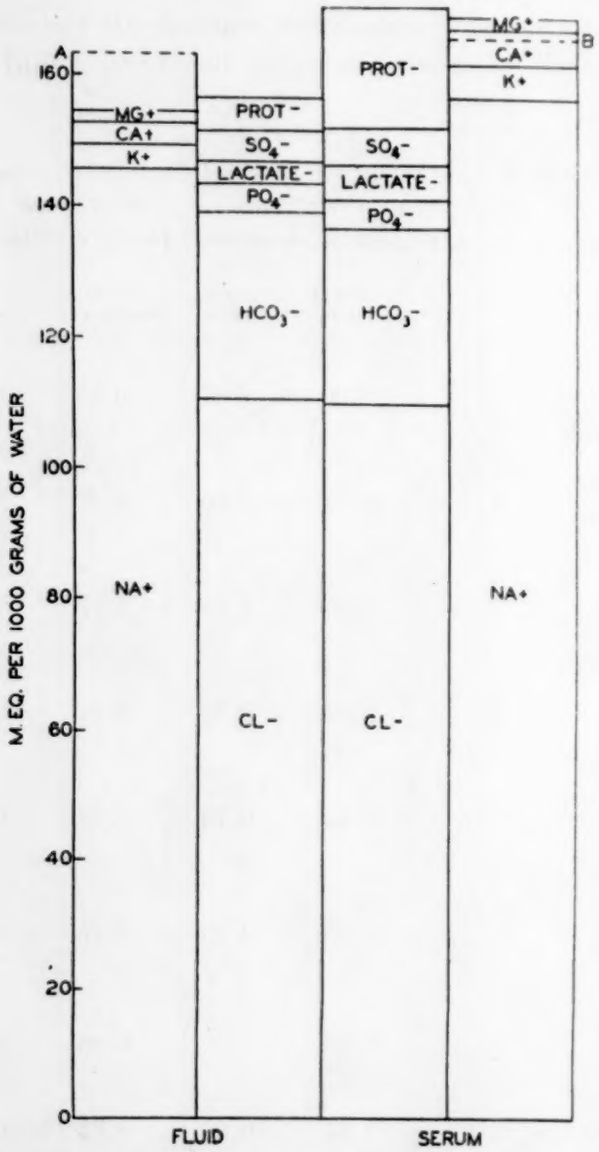

Fig. 1. The distribution of anions and cations between serum and normal cattle synovial fluid.

It will be noted that the summations of individual bases in the fluid and serum do not correspond exactly with the average determined total base values for fluid (A) and serum (B).

The formulae of Van Slyke, Hastings, Hiller and Sendroy (268) were used in estimating the proteinate. In the case of mucin, the base-binding power was assumed to be ten times the average base-binding power of albumin and globulin.

The average distribution ratio of the total inorganic phosphate is 1.00, essentially the same as that of chloride. The similarity of these ratios indicates that inorganic phosphate is entirely diffusible, and that its distribution is determined by the same laws of membrane equilibrium as regulate the distribution of chloride between serum and synovial fluid. This result is in accord with the findings of Brull (43), Heim (122) and Walker (271, 272), but in contrast to the conclusions of Greene and Power (106) and Gilligan, Volk and Blumgart (98).

The average ratio $\frac{(HCO_3)\,s}{(HCO_3)\,f}$ is 0.94, which is in close agreement with the theoretical Donnan ratio, and conforms fairly well with the bicarbonate ratio found for other fluids. Deviation from the chloride ratio may depend on several factors. The bicarbonate ratio represents that between arterial blood and fluid, and, as would be expected, this ratio has been found to be lower than that between venous blood and fluid. Furthermore, the discrepancy may be due to a difference between the carbon dioxide content of blood from the carotid artery and that of blood from capillaries around the knee. In addition, true equilibrium probably never exists because carbon dioxide is constantly being poured into the fluids from the tissues to be removed by the blood (204).

The average ratio $\frac{(\text{lactic acid})\,s}{(\text{lactic acid})\,f}$ is 2.11. The concentrations in individual cases show marked variations. The extremely high distribution ratio and the variations in concentrations in individual sera and fluids are presumably explicable as in the case of the sugar by the fact that the ante mortem struggle caused a sudden rise in the blood lactic acid which was not reflected in the joint fluid by the time of death.

The average ratio $\sqrt{\frac{(SO)_4\,s}{(SO)_4\,f}}$ is 1.06, 7 per cent higher than the chloride ratio. Since the determination of sulphate in blood and fluid is not exact, the 7 per cent deviation is not of great significance, and the sulphate ratio may be considered in general agreement with the chloride ratio.

The average ratio $\frac{(\text{total anions})\,s}{(\text{total anions})\,f}$ is 0.99, which agrees well with the ratio found for the "in vivo dialysate" (106), and with the chloride ratio.

The average ratio $\frac{(Na)\,f}{(Na)\,s}$ is 0.93, which is identical with the theoretical Donnan ratio, but slightly lower than the chloride ratio. It is in fairly good agreement with the ratios found by other workers. The slight deviation from the chloride ratio may indicate that a small percentage (6 per cent) of the sodium is held in the serum in a non-diffusible form, presumably bound to protein.

The average ratios $\frac{(K)\,f}{(K)\,s}$ (0.76) and $\sqrt{\frac{(Mg)\,f}{(Mg)\,s}}$ (0.88) were obtained from a smaller number of analyses, the results of which varied considerably. However, the deviation from the chloride ratio is great and of the same magnitude as that found for other fluids. One can conclude that part of the potassium (approximately 25 per cent) and part of the magnesium (approximately 30 per cent), as well as part of the calcium, are held in the serum in a non-diffusible state.

The average ratio $\frac{(\text{total base})\,f}{(\text{total base})\,s}$ (0.98) is identical with the chloride ratio. The results of the individual determinations of the total base concentration, however, varied markedly. The distribution ratio of total base concentrations obtained by summation of the average concentrations of the individual cations in the fluid and serum is 0.91. This value may be a more accurate indication of the base held in the serum in a non-diffusible state.

The average ratio $\sqrt{\frac{(Ca)\,f}{(Ca)\,s}}$ is 0.83, and indicates, as do the similar calcium ratios obtained for the "in vivo dialysate" and other fluids, that part of the calcium is held in the serum, presumably bound to protein. Of more significance than the distribution ratio of total calcium is that of ionized calcium. Calculation of the calcium ion in serum and fluid from the protein and total calcium concentrations (McLean and Hastings, 178) gives a ratio $\frac{(Ca^{++})\,f}{(Ca^{++})\,s}$ of 1.18. This ratio is much higher than would be expected from the laws of membrane equilibrium. The difference may be explicable in part by the fact that, in calculating the calcium ion concentrations of the serum and fluid, no consideration was given to the difference in pH and albumin-globulin ratios, but in larger part by the fact that the mucoprotein was included as part of the total protein and assumed to have the same effect as the serum proteins. That this is incorrect is evident from a comparison of the calcium concentration of synovial fluid with that of other body

fluids known to be dialysates of blood plasma. A review of the results reported on such fluids gives an average empirical ratio $\frac{(Ca)\ \text{dialysate}}{(Ca^{++})\ \text{serum}}$ of 1.33 (Hastings, 119). Using the average calcium ion concentration in the cattle serum (1.21 mM. per kgm. of water), the calcium concentration of synovial fluid calculated from the above empirical formula is 1.61 mM. per kgm. of water in contrast to the observed value of 1.90 mM. The difference (0.29 mM. per kgm. of water) represents an estimate of the calcium bound by mucin. In terms of millimols of calcium bound per gram of mucin the figure is 0.23 mM., a value approximately ten times that obtained for serum proteins (178). This is in accord with the results of experiments on mucin discussed above (265), which indicate that the base-combining power of mucin is high.

The distribution of electrolytes agrees, in general, with that expected from the Gibbs-Donnan theory of membrane equilibrium, and with the results obtained by Greene and Power (106) in the study of the "in vivo dialysate," and by various workers in the study of other fluids which have the composition of dialysates.

Theories of Origin. Many theories concerning the origin of synovial fluid have been proposed and will be presented in brief.

1. Synovial fluid is a product of glandular synovial membrane cells. This concept originated with Havers (121) and was supported by many subsequent workers (14, 32, 42, 64, 65, 102, 135, 149, 165, 179, 208, 236, 238, 252, 263). It was based chiefly on physiological deduction and gross examination. Drawings or photomicrographs of glandular cells have never been presented.

2. Synovial fluid is a mixture of the products of disintegration of synovial membrane and a transudate from the capillaries and lymphatics. This theory, presented by Frerichs in 1846 (91), has been supported in a modified form by other workers (4, 54, 78, 83, 110, 111, 118, 125, 169, 206, 210, 258). Histological studies served as the chief basis for such conclusions.

3. Synovial fluid is formed from the products of attrition of cartilage. Originally proposed by Ogston (197) and Banchi (18), this theory has received no support except for a statement by Fisher (83), in which he suggested that a portion of the synovial fluid mucin might be derived from articular cartilage as it becomes worn.

4. Synovial fluid consists of substances elaborated by synovial membrane cells with the addition of a transudate from the capillaries

and lymphatics (89, 151, 176, 229). This theory is for the most part based on histological studies with special stains.

5. Synovial fluid is the liquid matrix of the connective tissue lining an enlarged tissue space, the joint cavity (30, 62, 132, 144, 147). According to this theory, which is based on analogy suggested by embryological and histological studies (130), the synovial fluid mucin corresponds to the mucoid constituent of other connective tissues. According to Vaubel (270) mucin is the ground substance of synovial tissue.

6. Synovial fluid is a dialysate from the blood capillaries. This theory was first suggested by Bichat (34) (1812) who proved that the "glands" described by Havers were fat deposits and stated that synovial fluid is formed by "exhalation" of the blood capillaries. This concept has been supported by subsequent workers (8, 22, 35, 52, 71, 92, 124, 141). More recent reviews (204, 235) of the existing data on synovial fluid and the experimental evidence from this laboratory presented above have led to the conclusion that synovial fluid is in ready diffusion equilibrium with plasma. Except for the presence of mucin, albumin and globulin, it could, therefore, be considered a diffusate or a simple ultrafiltrate of serum.

The concept that synovial fluid is a dialysate of blood plasma to which mucin is added as the fluid diffuses through the connective tissue surrounding the joint is not fundamentally different from the theory that synovial fluid represents the liquid matrix of connective tissue, nor does it differ materially from that in which synovial fluid is considered a combination of synovial membrane cell products and a transudate from the capillaries.

The distribution of electrolytes and non-electrolytes between serum and normal synovial fluid is in accord with the concept that normal synovial fluid is a dialysate in equilibrium with blood plasma. This relationship is also suggested by the marked vascularity of the synovial intima whose numerous capillaries and venules are in close proximity to the articular lumen. Such a theory explains all known facts of the physical and chemical composition of synovial fluid except the presence of albumin, globulin and mucin. The presence of albumin and globulin can be ascribed presumably to slight capillary permeability to protein as discussed above. The presence of the mucin, whether it be formed by the surrounding connective tissue, as seems most likely, or by cartilage, in no way invalidates the theory.

Little is known concerning the source of synovial fluid mucin. Kling

(149) considers its presence, as determined by the "sac phenomenon," in both synovia and saliva as evidence of the secretory nature of synovial fluid. Kling failed to appreciate that the formation of a sac in acetic acid merely indicates the presence of a precipitable quantity of mucin and does not prove the secretory origin of synovial fluid. We have never observed glandular cells in synovial tissue and are not aware that microscopic evidence of such cells has been presented. In fact, histological studies as described above show that synovial membrane consists of modified connective tissue. The theory that mucin is formed in cartilage is supported only by a similarity of staining reactions (18). No chemical identity between mucin and any constituent of cartilage has been established. Other workers have described "granules" which have been interpreted as representing mucin in synovial membrane cells (54, 88, 149, 176, 229), but the staining methods employed are open to question. There is good evidence to suggest that synovial fluid mucin originates as the mucinous component of the connective tissue lining the joint (30, 62, 132, 140, 144, 147, 270). King (147) has offered the demonstration of Golgi apparatus in support of this theory. Extraction from the subcutaneous tissue of rabbits and the tissue lining the astragalotibial joints of cattle of a substance similar to synovial fluid mucin as shown by its physical properties and by enzymatic studies (218) confirms the suggestion that mucin is formed by the connective tissue cells surrounding the joint. Its entrance into the joint is made possible by the diffusion of plasma water from the underlying vessels through the subsynovial tissue and membrane.

The high colloidal osmotic pressure and high calcium concentration of the synovial fluid form the only essential differences between the synovia and other fluids with the composition of plasma dialysates. These properties of joint fluid are presumably due to the presence of mucin and indicate that mucin plays a rôle in the exchange of water and other substances between the vascular system and the joint cavity. The finding of similar, if not identical, mucins in subcutaneous tissue and synovial membrane suggests that mucin may have a similar action in all connective tissue fluid.

The functions of synovial fluid are partly those inherent in its position as intercellular connective tissue fluid and partly those associated with the specialized functions of joints. It is the main source of nourishment for the avascular articular cartilage and aids also in the nutrition of the superficial cells of the synovial membrane, particularly in villi

without blood supply. Bordier (39) suggests that it may act as an important cohesive force in the joint. Since Havers (121), the significance of synovial fluid as a lubricant has been generally acknowledged. Jones (139) devised an experiment to test articular lubrication. He used the proximal interphalangeal joint, the middle phalanx remaining fixed. Applying the Osborne-Reynolds law, and comparing load and friction, Jones concluded that fluid film lubrication is the usual form in human joints, but that some solid friction must occur when speed and/or eccentricity are insufficient to maintain a pressure film. The fluid film formed withstood a load of 900 pounds per square inch, a load that will crush bone.

Exchange of substances through the articular membrane. For obvious reasons the interest of workers has centered on the mechanism of exchange between the joint cavity and the body as a whole. First, because maintenance of the normal state and function of the joint requires entrance of nutritive material and removal of potentially toxic end products. Next, because the anatomical structure of the synovialis differs radically from that of the other body membranes across which transfer has been studied, as peritoneum, pleura and vascular endothelium. Finally, because in joint diseases, it frequently appears to be this phase of the physiology which is disrupted, early and to a striking degree.

Histological studies, as reviewed above, show that the joint cavity should be considered a large tissue space. The connective tissue enclosing it, although constituting an anatomical boundary, is not a true membrane such as the peritoneum, pleura and those of the choroid plexus and glomerulus. The contents of the articular cavity are presumably in direct communication with the matrix of the synovial tissues. Therefore, one can assume that exchange of substances between the vascular or lymphatic system and the synovial fluid involves the same processes as those governing the exchange in any connective tissue fluid. Such transfer necessitates passage through an endothelial wall as well as diffusion through the intercellular spaces of the synovial membrane. In the case of synovial membrane, the term, permeability, implies these two processes and will be used in this sense throughout the following presentation. Such permeability will be regulated by various physicochemical factors. It will depend on the capillary and lymphatic permeability, the charges of the diffusing substances and any oxidation-reduction potentials involved. As was pointed out above, it will also be subject to the laws of equilibrium

across a semipermeable membrane as formulated by the Gibbs-Donnan theory. The concentration of synovial fluid constituents will vary with their concentration in the plasma and will be influenced by the metabolic activity in the joint cavity.

Comparison of the composition of joint fluid with that of plasma indicates that non-electrolytes diffuse readily in either direction between blood and synovial fluid and that electrolytes are distributed in accord with the Gibbs-Donnan theory. Direct evidence of the mode of transfer across the synovial membrane has been obtained by studying the disposition of various substances following intravenous and intra-articular injection.

Thiocyanate and sugar diffuse readily into the joint spaces following intravenous injection in calves (285). The appearance time in the fluid varies; thiocyanate was detected ten minutes after injection, whereas the glucose concentration showed no rise for twenty minutes. A similar twenty-minute lag was apparent when the blood sugar was falling. The difference between thiocyanate and sugar is significant since both substances are of small size and freely diffusible and it emphasizes the multiplicity of factors involved in transfer.

Thiocyanate diffusion equilibrium between joint fluid and serum was attained in from one to five hours. In the experiments of Lavietes, Bourdillon and Klinghoffer (166), equilibrium was reached in one-half to one hour, but it was determined by stable blood concentrations rather than by true diffusion equilibrium. In both series of experiments the concentration of thiocyanate in the fluids averaged approximately 9 per cent lower than that of the blood, indicating that part of the thiocyanate is held in the blood in a non-diffusible state.

The concentration of thiocyanate in the aqueous humor was much lower than that in the synovial fluid, and only traces were present in the cistern fluid. The differences indicate anatomical and physiological variations in the barriers and are similar to those found in protein studies discussed below.

Readily diffusible substances of small molecular dimensions in homogeneous solution are removed from joints primarily by way of the subsynovial blood capillaries, as shown by the investigations of Rhinelander, Bennett and Bauer (216). In their experiments, absorption of mecholyl after intra-articular injection was measured either directly by its effect on the blood pressure, or indirectly by biological assay of the joint washings. Mecholyl in aqueous solution was readily absorbed from all joints. The effect on blood pressure was evident

within thirty to sixty seconds after injection. Passive exercise increased strikingly the rate of absorption, presumably by raising the intra-articular pressure and augmenting the flow of blood. Absorption was slightly increased from mildly inflamed joints but was definitely greater from those in which severe, acute inflammation had been produced. This was true of both exercised and resting joints. Intra-articular injection of adrenalin completely prevented absorption of mecholyl, presumably by constricting the subsynovial capillaries. Aqueous solutions of pituitrin and pilocarpine also were readily removed from joints by way of the blood stream. Determinations on thoracic duct lymph showed that little if any mecholyl was absorbed by way of the subsynovial lymphatics.

Intra-articular injection of dyes has given further evidence of the mode of absorption of diffusible substances of relatively small size. The uniform result of these experiments, as first formulated by Braun (40) is that such substances pass rapidly into the intercellular spaces of the membrane by diffuse penetration to variable depths (83, 260). The subsequent route of removal, however, is debated. Tillmanns (260) described the collection of dye in the venules of the synovial membrane. Although Braun (40), Tillmanns (260), Rynearson (228) and Fisher (83) found varying amounts in the lymphatics, the consensus of opinion (83, 158, 228, 260) is that diffusible dyes are absorbed largely by capillaries and only slightly by lymphatics. Allen (6), on the other hand, concluded that removal of diffusible dyes takes place entirely by way of the lymphatics. Since his experiments were performed on dead animals perfused with saline, the conclusions are not valid.

Experiments in which potassium or sodium iodide has been determined in urine or saliva at various intervals after intra-articular injection (53, 83, 199) are less indicative of the mode or rate of absorption because these salts cause marked inflammation of the synovial membrane and are readily stored in the organism. The amount of phenolsulphonephthalein remaining in the knee joints of normal dogs at the end of an hour has been determined (50). While it is impossible to draw conclusions from these experiments as to the mode of absorption, they afford a basis of comparison for similar studies in joint diseases.

The rapid rate of diffusion of salts from the joint cavity and from the capillaries into the synovial membrane is apparent from the experiments of Mayeda (176). After injecting sodium salicylate intravenously in rabbits, he introduced an iron salt into the joint cavity

and could demonstrate ferric oxide salicylate in the membrane ten minutes after injection.

Permeability of the synovial membrane to substances of large molecular size, such as proteins, is of special significance in the physiology of joints because of their effect on osmotic pressure, and thereby on water exchange. The entrance and removal of colloids involves a more complex mechanism than the exchange of readily diffusible small molecules. The presence of albumin and globulin in normal synovial fluid indicates presumably that the capillaries are slightly permeable to proteins. The high albumin-globulin ratio in the fluid suggests a greater permeability to albumin than to globulin in corroboration of the findings of other investigators in lymph, edema and ascitic fluid (73, 80, 100, 277, 279). Experimental studies in this laboratory (27) have given further knowledge of the permeability of the synovial membrane to proteins. Egg albumin was found in the knee joint of rabbits within five minutes after intravenous injection. The foreign protein concentration in the joint fluids tended to vary directly with the concentration in the serum. Differences were encountered, however, between individual animals and occasionally between corresponding joints of the same animal. Such individual variations represent local, and presumably temporary, changes in the permeability of the membrane. The appearance time of larger molecules, horse serum albumin and euglobulin was approximately twenty minutes longer than that of egg albumin. In experiments of twenty-four hours' duration or longer, the concentration in the joints tended to increase, indicating that the rate of removal is slower than the rate of entrance. The fact that the globulin concentration increased sooner than that of albumin suggests that globulin is removed more slowly.

Examination of other body fluids in the above experiments showed marked differences in the ease with which the foreign proteins passed from the vascular system into synovial fluid, aqueous humor, spinal fluid and urine. The proteins appeared in aqueous humor at approximately the same time as in synovial fluid, but were present in lower concentrations in the former. In the experiments of longer duration there was no tendency to accumulation of the proteins in the aqueous humor, suggesting that the rate of removal approached the rate of entrance. In spinal fluid the proteins appeared only rarely and in minimal amounts, indicating that the choroid plexus is less permeable to these substances. Egg albumin was eliminated readily by the kidney. The horse serum proteins appeared only occasionally in the

urine, and their concentrations in the blood remained relatively high for several days. The variation in permeability between the different membranes was shown to be dependent both on the molecular size of the proteins and on the anatomical and physiological character of the intervening barriers. With the exception of the kidney glomeruli in the case of egg albumin, the synovial membrane is the most permeable. This greater permeability and the slow rate of removal probably explain the higher total protein content of normal synovial fluid in comparison with aqueous humor and spinal fluid.

Proteins, in contrast to small, readily diffusible substances, are removed from joints only by way of the lymphatics. This mode of removal of colloids was first suggested by Böhm (37) who noted fat globules in the inguinal lymph nodes twenty-four hours after injection of milk into the knee joint. Subsequently, lymphatics were outlined after intra-articular injections of colored mucilage (83), a colloidal suspension of silver (83) and a mixture of turpentine, dye and ether (246). Conclusive evidence that proteins are removed only by the lymphatics was obtained in the investigations of Bauer, Short and Bennett (20). Egg albumin and horse serum albumin were found in thoracic duct lymph thirty minutes after injection into the knee joints of dogs if the legs were passively exercised or massaged. None of the injected protein appeared in the blood stream when all communications between the lymphatic and vascular systems were eliminated. Horse serum globulin was removed from joints with difficulty, if at all, even when the leg was exercised.

The removal of particulate matter such as carbon particles, bacteria and blood cells is achieved by a combination of several distinct processes. It takes place more slowly and is less complete than the removal of soluble substances. The earliest experiment, that of Böhm with cinnabar (37), was inconclusive but von Mosengeil (183) detected India ink in all lymphatics between knee joint and inguinal nodes two minutes after intra-articular injection and joint massage. The rapidity of spread recorded here is difficult to reconcile with the results of subsequent workers, unless one assumes that the membrane ruptured or leakage occurred at the site of puncture. Braun (40), on the basis of careful histological study, described the steps in the removal of the carbon particles of India ink. The increased intra-articular pressure caused a large part of the material to enter directly into the intercellular spaces of the membrane. Another part was removed by connective tissue phagocytes and to a smaller extent by polymorphonuclear

leukocytes emigrating from the membrane and carrying the substance back into it. A third part remained in the joint cavity to be ultimately organized. A small portion was taken up by the synovial cells bordering directly on the joint space. From all of these sources, by repeated phagocytosis, some carbon finally reached the regional and distant lymphatics. More recent investigations (143, 157, 159, 176, 228, 247), some of which were done without knowledge of Braun's work, have led to strikingly similar conclusions. Mayeda (176) pointed out that the intensity of resorption differs in the various parts of the membrane, according to anatomical structure. In Key's outstanding report (143), the wide ultimate distribution of carbon in bone-marrow, liver and spleen is described.

The value of joint exercise, particularly passive, and of massage for increasing absorption from joints has been demonstrated repeatedly (6, 20, 40, 157, 183, 187, 189). It is probable that the increase is achieved mainly by augmenting the flow of blood and lymph and not merely by elevating the intra-articular pressure (251, 260). The effect of compression of the joint on absorption has not been conclusively established. Tillmanns (260) reported increased absorption of a water-soluble dye. Kroh (157) and Müller (189) concluded that compression made no appreciable difference in resorption of particulate matter. The results do no more than suggest that compression increases absorption by way of the capillaries and does not affect removal by the lymphatics.

The entrance of particulate matter into the joint has been less thoroughly investigated. Kuhns and Weatherford (159) found that carbon particles injected into the abdominal wall of rats appeared in the histiocytes of the synovial membrane, but in smaller quantities than in the liver, spleen, kidney and bone marrow. Experimental studies (61, 68, 69, 180, 223, 244) have shown that living bacteria gain access more readily to synovial fluid than to spinal fluid, aqueous humor and urine. The relatively greater permeability of the synovial tissue as compared with true membranes is again apparent.

Alterations in physiology produced by disease. While a consideration of articular disorders and of the anatomical changes produced thereby is not within the scope of this discussion, certain features of pathological joint physiology serve as an adjunct to our understanding of normal joint function. The changes produced by disease depend on two fundamental alterations in function: altered permeability of synovial tissue and disturbance of intra-articular metabolism.

The former permits increased entrance of water, readily diffusible substances, proteins including fibrinogen, leukocytes, antibodies and presumably enzymes. The distribution of diffusible substances follows the laws that apply to normal joints. In the case of a utilizable material, such as sugar, equilibrium is established, but with increased consumption, as in the case of septic joints, the fluid sugar is markedly decreased (8, 52, 62, 141, 191, 221). The low sugar content in some cases of rheumatoid arthritis, on the other hand, is due, at least in part, to decreased permeability of the membrane to sugar (266). Transfer of thiocyanate into these same joints is essentially normal and entrance of proteins is increased. The mechanism of this differential permeability is not understood, but the experimental results emphasize again that a variety of factors is involved in the transfer. The amount of protein entering the joint space varies directly with the degree of inflammation. It is slightly greater than normal in traumatic joints and markedly increased in severe rheumatoid and septic arthritis (221). The proportion of globulin in the entering protein increases as the membrane becomes more permeable, and therefore the albumin-globulin ratio more closely approaches that of serum.

Alterations in the membrane lead also to diminished removal of colloidal and particulate matter. Experimental evidence suggests that this is due to a decreased number of synovial lymphatics (158, 201). The reduced absorption, combined with greater entrance, may account for the increasing protein concentration and decreasing albumin-globulin ratio in rheumatoid effusions of long duration (221). The abnormally high concentration of colloidal material raises the osmotic pressure and results in persistence of the effusion.

The altered metabolism of the joint is most apparent in the case of sugar and mucin. Greater utilization of sugar results from various factors: increased cellular activity of fixed tissue cells, a larger number of leukocytes, an enhanced enzymatic activity and bacteria, if present. Greater metabolic requirement for sugar, coupled with decreased supply, may lower the fluid sugar to a level insufficient for adequate nutrition of cartilage (266). In the case of mucin both formation and destruction are affected. In traumatic effusions the unit concentration of mucin remains normal despite a great increase in fluid volume (221). Whether the inflammation produced by trauma stimulates formation of mucin by the connective tissue cells or merely increases the amount of mucin carried into the joint has not been established.

Infectious fluids show a decreased concentration of mucin, a reduced viscosity and an atypical precipitate with acetic acid. The similarity of these changes to those produced by the bacterial enzyme, mucinase (218), suggests that increased destruction of mucin takes place rather than decreased formation. Such fluids also show an increase in the normal difference between the total glucosamine and the glucosamine of the precipitated mucin, again indicating accelerated breakdown of mucin.

JOINTS AS STRUCTURAL AND FUNCTIONAL UNITS. Finally a synthesis of the evidence will be attempted by integrating the individual results into a working scheme. We may thereby test their consistency and become aware of missing links.

Articulations serve the rather primitive purposes of motion and weight-bearing. Their structure, accordingly, is simple and firm. Cartilage in terms of comparative zoölogy is the oldest tissue in the human body (169) and all principal joint components have a common mesenchymal origin. The articular capsule, which consists of tough, fibrous and modified connective tissue, bounds with the epiphyseal cartilages a potential space partly filled with a hypocellular fluid of high viscosity. Articular construction allows ease of motion to be combined with relatively great stability. The viscous synovia aids by its cohesive action in uniting the articular ends and forms a strong fluid film upon which the cartilaginous surfaces glide with negligible friction. The capsule and tendons inserting into it, the ligaments and muscular tone, impart steadiness to the joint, while cartilage by virtue of its remarkable elasticity is able to buffer the impacts to which the rigid skeletal system is exposed. Cartilage under normal conditions and particularly with advancing age is subject to wear and tear in a measure unequalled by any other tissue, with the exception of the integument. The resistance thus required excludes fragile and sensitive structures from the architecture of this tissue and may explain the disappearance of vascular and nervous elements at the time of birth. Hence the usual avenues of metabolic exchange are not available for articular cartilage and its subsistence depends largely, if not altogether, on the synovial fluid. We can merely suggest the manner in which metabolites enter and leave the cartilaginous substance, by pointing to its sponge-like resilience and the ability of dyes to diffuse along the intercellular system of fibrils. Since arterial blood does not come in contact with cartilaginous cells, the breakdown of glycogen must take place through anaerobic oxidation. This is confirmed by

the low respiratory quotient of this tissue. The histological picture characterized by a notable predominance of matrix over cells reflects both the physical requirements made upon cartilage and the disadvantageous metabolic conditions under which it functions.

Since the synovia conveys to cartilage its means of existence, the nature and origin of that fluid are of prime physiological importance. The distribution of electrolytes and non-electrolytes between blood and synovia and the marked vascularity of the synovial membrane indicate that the fluid is a dialysate of blood plasma. It contains an admixture of albumin, globulin and mucin. The presence of albumin and globulin is probably due to slight capillary permeability. The origin of mucin remains uncertain, but the evidence to date suggests that it is formed by the connective tissue cells of the synovialis and carried into the joint by the plasma dialysate as it diffuses through the synovial tissues. The factors influencing the formation of the synovial fluid require further investigation. Under normal conditions, the opposing forces of capillary pressure and osmotic pressure difference between plasma and fluid determine the volume of fluid present. Substances entering the joint from the systemic circulation must pass the capillary endothelium and diffuse through the interstitium of the synovialis. The concept of synovial tissue as a membrane analogous to the true body membranes should be discarded because uninterrupted continuity exists between the intercellular fluid of the synovialis and the synovia. Compared to true membranes, synovial tissue has a markedly greater permeability. Resorption of small molecules takes place almost entirely by the blood vascular system and only to a slight degree through the lymphatics, while the larger protein molecules are removed with difficulty and only by way of the lymphatics. The predominant cellular constituents of the synovial fluid are mononuclear phagocytes. They carry particulate matter and the cellular debris of wear and tear from the joint into the lymphatics in a manner which suggests that the synovial membrane shares in reticulo-endothelial activity. Balance of all the factors mentioned must obtain for the maintenance of a normal amount and composition of synovial fluid in the joint cavity. The slightly negative intra-articular pressure, varying with motion, would also appear to play a rôle in this equilibrium. The possible influence of the autonomic nervous system through its effect on blood vessels and cellular activity warrants further study.

Deviations from normal articular physiology, whatever their cause, consist chiefly of alterations in the synovial tissue and changes in

intra-articular metabolism. While the former leads to quantitative and qualitative disturbances of the exchange equilibrium, the latter results mainly in deficient supply of nutriment to cartilage. It may also reduce the available mucin to which synovia owes important physiological properties. Cartilage, once injured, shows little or no tendency to regenerate and repair takes place by invading fibrous tissue through marginal or subchondral proliferation.

The resemblance of the cytological characteristics of synovial fluid to those of tissue fluid, the similarity of its composition to that of other plasma dialysates, the presumptive identity of synovial fluid mucin with that obtained from subcutaneous tissue, lead to the conclusion that synovial fluid is an example of tissue fluid in general. Likewise, the embryological derivation of the joint cavity from a mesenchymal cleft, the histological and physiological properties of the synovialis, its great reparative and proliferative power and the nature of its cytological response to injury, all indicate that the articular lumen represents a connective tissue space.

REFERENCES

(1) Achard, C. and M. Piettre. Compt. rend. Acad. d. Sc. **191**: 1412, 1930.
(2) Achard, C. and M. Piettre. Compt. rend. Acad. d. Sc. **192**: 996, 1931.
(3) Achard, C. and M. Piettre. Compt. rend. Acad. d. Sc. **194**: 221, 1932.
(4) Aeby, C. Ein Lehrbuch der Anatomie. Leipzig, Vogel: 1868–1871. Cited by Mayeda.
(5) Albert, E. Wien. mathem. naturwiss. Sitzungsber. **64**: Abt. 2, 1871.
(6) Allen, A. L. South African J. Med. Sc. **1**: 31, 1935.
(7) Allen, S. W. Boston Med. and Surg. J. **148**: 388, 1903.
(8) Allison, N., F. Fremont-Smith, M. F. Dailey and M. Kennard. J. Bone and Joint Surg. **8**: 758, 1926.
(9) Allison, N. and R. K. Ghormley. Diagnosis in joint disease. William Wood & Co., New York, 1931.
(10) Amprino, R. Ztschr. f. Zellforsch. u. mikr. Anat. **28**: 734, 1938.
(11) Amprino, R. and A. Bairati. Ztschr. f. Zellforsch. u. mikr. Anat. **20**: 143, 1933.
(12) Archangelsky. Zentralbl. f. d. med. Wissensch. **6**: 658, 1868.
(13) Arnold, R. M. and L. B. Mendel. J. Biol. Chem. **72**: 189, 1927.
(14) Aschoff, L. Pathologische Anatomie. II. G. Fischer, Jena, p. 233, 1911.
(15) Axhausen, G. Arch. klin. Chir. **124**: 543, 1923.
(16) Axhausen, G. Virchow's Arch. **252**: 458, 1924.
(17) Bär, E. Arch. f. Entwcklngsmechn. **108**: 739, 1926.
(18) Banchi, A. Sperimentale **55**: 273, 1901.
(19) Bauer, W., G. A. Bennett, A. Marble and D. Claflin. J. Exper. Med. **52**: 835, 1930.
(20) Bauer, W., C. L. Short and G. A. Bennett. J. Exper. Med. **57**: 419, 1933.
(21) Baum, H. Anat. Hefte **44**: 439, 1911.

(22) BÉCLARD, P. A. Éléments d'anatomie générale. Paris, Asselin: 1865, 4th ed. Cited by HAMMAR AND MAYEDA.
(23) BENNETT, G. A. AND W. BAUER. J. Bone and Joint Surg. **17:** 141, 1935.
(24) BENNETT, G. A. AND W. BAUER. Am. J. Path. **9:** 951, 1933.
(25) BENNETT, G. A. AND W. BAUER. Unpublished.
(26) BENNETT, G. A., W. BAUER AND S. J. MADDOCK. Am. J. Path. **8:** 499, 1932.
(27) BENNETT, G. A. AND M. F. SHAFFER. J. Exper. Med. **70:** 277, 1939.
(28) BENNINGHOF, A. Ztschr. f. Anat. u. Entwcklngsgesch. **75:** 43, 1925.
(29) BENNINGHOF, A. Ztschr. f. Zellforsch. u. mikr. Anat. **2:** 783, 1925.
(30) BERGER, L. Am. J. Cancer **34:** 501, 1938.
(31) BERNAYS, A. Morphol. Jahrb. **4:** 403, 1878.
(32) BERNSTEIN, J. Lehrbuch der Physiologie des thierischen Organismus, im Speciellen des Menschen. Stuttgart, Enke 1894. Cited by MAYEDA.
(33) BICHAT, X. Traité des membranes en général et des diverses membranes en particulier. Paris, Brosson, Gabon Cie. 1799.
(34) BICHAT, X. Anatomie Générale, Appliquée à La Physiologie et à La Médecine. Paris, 4, part 2, p. 538, 1812.
(35) BICK, E. M. J. Bone and Joint Surg. **12:** 33, 1930.
(36) BIER, A. Deutsch. med. Wchnschr. **45:** 225, 1919.
(37) BÖHM, R. Dissertation. Würzburg, Becker: 1868. Quoted by FICK.
(38) BOOTS, R. H. AND G. E. CULLEN. J. Exper. Med. **36:** 405, 1922.
(39) BORDIER, H. Presse méd. **44:** 1257, 1936.
(40) BRAUN, H. Deutsch. Ztschr. f. Chir. **39:** 35, 1894.
(41) BRAUS, H. Arch. f. Entwcklngsmechn. d. Organ. **30:** 459, 1910.
(42) BRINTON, W. In R. B. TODD, Cyclopaedia of anatomy and physiology. London, 1847–49, 4, part 1, p. 511. Cited by HAMMAR.
(43) BRULL, L. Arch. internat. de physiol. **32:** 138, 1930.
(44) BURCKHARDT, H. Beitr. z. klin. Chir. **130:** 163, 1924.
(45) BÜRGER, M. AND E. MÜLLER. Ztschr. f. d. ges. exper. Med. **25:** 345, 1921.
(46) BUTLER, A. M. AND H. MONTGOMERY. J. Biol. Chem. **99:** 173, 1932.
(47) BYWATERS, E. G. L. J. Path. and Bact. **44:** 247, 1937.
(48) BYWATERS, E. G. L. Nature **138:** 30, 1936.
(49) BYWATERS, E. G. L. Nature **138:** 288, 1936.
(50) BYWATERS, E. G. L., N. RUDO AND W. BAUER. Unpublished.
(51) CAJORI, F. A., C. Y. CROUTER AND R. PEMBERTON. Arch. Int. Med. **37:** 92, 1926.
(52) CAJORI, F. A. AND R. PEMBERTON. J. Biol. Chem. **76:** 471, 1928.
(53) CECCA, R. Clin. Chir. Milano **15:** 1203, 1907.
(54) CHERRY, J. H. AND R. K. GHORMLEY. J. Bone and Joint Surg. **20:** 48, 1938.
(55) CHURCHILL, E. D., F. NAKAZAWA AND C. K. DRINKER. J. Physiol. **63:** 304, 1927.
(56) CIOCIOLA, F. Policlinico **28:** 229, 1921.
(57) CLERMONT, D. Compt. rend. Assoc. des Anat. 10 Réunion. Marseilles, p. 155, 1908.
(58) CODMAN, E. A. The shoulder. Thomas Todd Co., Boston, 1934.
(59) COGGESHALL, H. C., G. A. BENNETT, C. F. WARREN AND W. BAUER. Unpublished.
(60) COGGESHALL, H. C., C. F. WARREN AND W. BAUER. Unpublished.

(61) Cole, R. I. J. Infect. Dis. **1:** 714, 1904.
(62) Collins, D. H. J. Path. and Bact. **42:** 113, 1936.
(63) Conklin, R. Am. J. Physiol. **95:** 98, 1930.
(64) Cornil, A. V. and L. Ranvier. Manuel d'histologie pathologique. Paris, 3d ed., p. 38, 1901.
(65) Cruveilhier, J. Anatomie déscriptive, Paris, Bechet: **4:** 818, 1834.
(66) Danckelman, A. v. Deutsch. Ztschr. f. Chir. **248:** 723, 1937.
(67) Darrow, D. C., E. B. Hopper and M. K. Cary. J. Clin. Investigation **11:** 701, 1932.
(68) Davis, D. J. J. Infect. Dis. **10:** 148, 1912.
(69) Davis, D. J. J. A. M. A. **58:** 1283, 1912.
(70) Dickens, F. and H. Weil-Malherbe. Nature **138:** 125, 1936.
(71) Drechsel, E. Herrmann-Handbuch der Physiologie **5:** 1883.
(72) Drinker, C. K. and M. E. Field. Am. J. Physiol. **97:** 32, 1931.
(73) Drinker, C. K., M. E. Field, J. W. Heim and O. C. Leigh, Jr. Am. J. Physiol. **109:** 572, 1934.
(74) Elliott, H. C. Am. J. Anat. **58:** 127, 1936.
(75) Fasoli, G. Arch. per le Sc. med. **29:** 365, 1905.
(76) Fasoli, G. Zentralbl. f. allg. Path. u. path. Anat. **16:** 257, 1905.
(77) Fick, R. Arch. f. Anat. u. Entwcklngsgesch., p. 391, 1890.
(78) Fick, R. Handbuch der Anatomie des Menschen. Anatomie und Mechanik der Gelenke. G. Fischer, Jena, 1904-11, 3 volumes.
(79) Field, M. E. and C. K. Drinker. Am. J. Physiol. **97:** 40, 1931.
(80) Field, M. E., O. C. Leigh, Jr., J. W. Heim and C. K. Drinker. Am. J. Physiol. **110:** 174, 1934-35.
(81) Fischer, K. Ztschr. f. d. ges. Anat. **87:** 746, 1928.
(82) Fisher, A. G. T. Brit. J. Surg. **8:** 493, 1921.
(83) Fisher, A. G. T. Chronic arthritis. The Macmillan Co., New York, 1929.
(84) Fisher, A. G. T. Lancet **2:** 541, 1923.
(85) Forkner, C. E. J. Exper. Med. **52:** 279, 1930.
(86) Forkner, C. E. J. Lab. and Clin. Med. **15:** 1187, 1930.
(87) Forkner, C. E., A. R. Shands, Jr. and M. A. Poston. Arch. Int. Med. **42:** 675, 1928.
(88) Franceschini, P. Monitore zool. It. **40:** 367, 1929.
(89) Franceschini, P. Arch. ital. di. Anat. **27:** 76, 1929.
(90) Fremont-Smith, F. and M. E. Dailey. J. Biol. Chem. **70:** 779, 1926.
(91) Frerichs, F. T. In R. Wagner. Handwörterbuch der Physiologie. Braunschweig **3:** Abt. 1, 463, 1846.
(92) Frey, H. Compendium of histology. Putnam, New York, 1876. (Translated by George R. Cutter.)
(93) Gendre, H. Bull. d'hist. appl. à la phys. **15:** 165, 1938.
(94) Gerken, N. A. Anat. Hefte **7:** Abt. 1, 21, 1896.
(95) Gerneck, I. Ztschr. Anat. **97:** 515, 1932.
(96) Gies, T. Deutsch. Ztschr. f. Chir. **18:** 8, 1882.
(97) Gilligan, D. R., M. C. Volk and H. L. Blumgart. New England J. Med. **210:** 896, 1934.
(98) Gilligan, D. R., M. C. Volk and H. L. Blumgart. J. Clin. Investigation **13:** 365, 1934.

(99) GOECKE, F. Elastizitätsstudien am jungen u. alten Gelenkknorpel. Orthop. Congress 1927. Quoted by MÜLLER.
(100) GOETTSCH, E. AND F. E. KENDALL. J. Biol. Chem. **109**: 221, 1935.
(101) GOLLWITZER-MEIER, K. Ztschr. f. d. ges. exper. Med. **46**: 15, 1925.
(102) GOSSELIN, L. A. Mém. acad. de méd. **16**: 367, 1852. Cited by MAYEDA.
(103) GOVAERTS, P. Bull. Acad. roy. de méd. de Belgique **7**: 356, 1927.
(104) GRANT, J. C. Brit. J. Surg. **18**: 636, 1931.
(105) GREENE, C. H., J. L. BOLLMAN, N. M. KEITH AND E. G. WAKEFIELD. J. Biol. Chem. **91**: 203, 1931.
(106) GREENE, C. H. AND M. H. POWER. J. Biol. Chem. **91**: 183, 1931.
(107) GURLT, E. J. Beiträge zur vergleichenden pathologischen Anatomie der Gelenkkrankheiten. G. Reimer, Berlin, 1853.
(108) GUSSENBAUER, C. Arch. f. Klin. Chir. **12**: 791, 1871.
(109) HÄBLER, C. Beitr. z. klin. Chir. **134**: 602, 1925.
(110) HAGEN-TORN, O. Arch. f. mikr. Anat. **21**: 591, 1882.
(111) HAMMAR, J. A. Arch. f. mikr. Anat. **43**: 266, 813, 1894.
(112) HAMMARSTEN, O. Jahresb. ü. d. Fortschr. d. Thierchem. **12**: 480, 1881–82.
(113) HAMMARSTEN, O. Lehrbuch der physiologischen Chemie, p. 105, 1891.
(114) HARBIN, M. AND A. R. MORITZ. Arch. Surg. **20**: 885, 1930.
(115) HARE, T. AND H. COHEN. Proc. Roy. Soc. Med. **22**: 1121, 1929.
(116) HARPUDER, K. Biochem. Ztschr. **169**: 308, 1926.
(117) HARRIS, H. A. Nature **130**: 996, 1932.
(118) HARTMANN, R. Handbuch der Anatomie des Menschen für Studierende und Aerzte. Strassburg, R. Schultz u. Co. 1881. Cited by MAYEDA.
(119) HASTINGS, A. B. Unpublished data.
(120) HASTINGS, A. B., H. A. SALVESEN, J. SENDROY, JR. AND D. D. VAN SLYKE. J. Gen. Physiol. **8**: 701, 1927.
(121) HAVERS, C. Osteologia nova. London, S. Smith, 1691. Quoted by SOUBBOTINE.
(122) HEIM, J. W. Am. J. Physiol. **103**: 553, 1933.
(123) HENLE, J. Arch. f. Anat. Physiol. u. wissensch. Med., p. 103, 1838.
(124) HENLE, J. Allgemeine Anatomie, Lehre von den Mischungs- und Formbestandtheilen des menschlichen Körpers. Leopold Voss, Leipzig, p. 385, 1841.
(125) HILDEBRAND, O. Deutsch. Ztschr. f. Chir. **42**: 292, 1896.
(126) HIS, W. Die Häute und Höhlen des menschlichen Körpers. Basel, Schweighauser, 1865.
(127) HOFFMANN, A., G. LEHMANN AND E. WERTHEIMER. Pflüger's Arch. **220**: 183, 1928.
(128) VON HOLST, G. Ztschr. f. physiol. Chem. **43**: 145, 1904.
(129) HORIYE, K. Virchow's Arch. **251**: 649, 1924.
(130) HUECK, W. Beitr. z. path. Anat. u. z. allg. Pathol. **66**: 330, 1919–20.
(131) HUETER, C. Virchow's Arch. **25**: 572, 1862; **26**: 484, 1863.
(132) HUETER, C. Virchow's Arch. **36**: 25, 1866.
(133) HULTKRANZ, J. W. Anat. Anz. Ergänzungsband, 1898.
(134) HUNTER, W. Phil. Soc. Trans. **42**: 514, 1743.
(135) HYRTL, J. Lehrbuch der Anatomie des Menschen, mit Rücksicht auf physiologische Begründung und praktische Anwendung. Braumüller, Wien, p. 134, 1878.

(136) Ikuta, A. Fukuoka-Ikwadaigaku-Zasshi **28:** 25, 1935.
(137) Ishido, B. Virchow's Arch. **244:** 424, 1923.
(138) Ito, L. K. Brit. J. Surg. **12:** 31, 1924.
(139) Jones, E. S. Lancet **230:** 1043, 1936.
(140) Jones, H. T. J. Bone and Joint Surg. **12:** 45, 1930.
(141) Keefer, C. S., W. K. Myers and W. F. Holmes, Jr. Arch. Int. Med. **54:** 872, 1934.
(142) Key, J. A. J. Bone and Joint Surg. **23:** 793, 1925.
(143) Key, J. A. J. Bone and Joint Surg. **8:** 666, 1926.
(144) Key, J. A. Anat. Rec. **40:** 193, 1928.
(145) Key, J. A. In Special cytology, II. The synovial membrane of joints and bursae. Paul B. Hoeber, Inc., New York, p. 735, 1928.
(146) Key, J. A. J. Bone and Joint Surg. **13:** 725, 1931.
(147) King, E. S. J. J. Path. and Bact. **41:** 117, 1935.
(148) Kling, D. H. J. Bone and Joint Surg. **12:** 867, 1930.
(149) Kling, D. H. Arch. Surg. **23:** 543, 1931.
(150) Koch, H. Deutsch. Ztschr. f. Chir. **201:** 367, 1927.
(151) von Kölliker, A. Mitt. d. Naturforsch. Gesell. Heft **1:** 93, 1849.
(152) König, F. Arch. Klin. Chir. **59:** 49, 1899.
(153) König, F. Arch. Klin. Chir. **124:** 1, 1923.
(154) König, F. Osteochondritis dissecans. 50 Chir. Kongress, 1926.
(155) Krause, W. Zentralbl. f. d. med. Wissensch. **20:** 211, 1874.
(156) Krogh, A., E. M. Landis and A. H. Turner. J. Clin. Investigation **11:** 63, 1932.
(157) Kroh, F. Deutsch. Ztschr. f. Chir. **94:** 215, 1908.
(158) Kuhns, J. G. Arch. Surg. **27:** 345, 1933.
(159) Kuhns, J. G. and H. L. Weatherford. Arch. Surg. **33:** 68, 1936.
(160) Kuwabara, G. J. Biochem. **16:** 389, 1932.
(161) Labor, M. and E. von Balogh. Wien. klin. Wchnschr. **32:** 535, 1919.
(162) Landis, E. M. Physiol. Rev. **14:** 404, 1934.
(163) Landis, E. M., L. Jonas, M. Angevine and W. Erb. J. Clin. Investigation **11:** 717, 1932.
(164) Landzert, A. Zentralbl. f. d. med. Wissensch. **5:** 369, 1867.
(165) von Langer, C. Lehrbuch der systematischen und topographischen Anatomie. Wien, 1885. Cited by Mayeda.
(166) Lavietes, P. H., J. Bourdillon and K. A. Klinghoffer. J. Clin. Investigation **15:** 261, 1936.
(167) Leidy, J. Am. J. Med. Sc. **17:** 277, 1849.
(168) Loeb, R. F., D. W. Atchley and W. W. Palmer. J. Gen. Physiol. **4:** 591, 1922.
(169) Lubosch, W. Bau und Entstehung der Wirbeltiergelenke. G. Fischer. Jena, 1910.
(170) Ludwig, C. F. W. and F. Schweigger-Seidel. Die Lymphgefässe der Fascien und Sehnen. Leipzig, Hirzel, 1872.
(171) Luschka, H. Arch. f. Anat., Physiol. u. wissensch. Med., p. 481, 1855.
(172) Magnus, G. Deutsch. Ztschr. f. Chir. **182:** 325, 1923.
(173) Malmejac, F. Bull. Soc. Chimie Biol. **4:** 190, 1922.
(174) Man, E. B. and J. P. Peters. J. Clin. Investigation **12:** 1031, 1933.
(175) Marquort, W. Ztschr. f. Zellforsch. **12:** 34, 1931.

(176) MAYEDA, T. Mitt. a. d. Med. Fakult. d. K. Univ. zu Tokio. **23:** 393, 1919–20.
(177) MCEWEN, C. J. Clin. Investigation **14:** 190, 1935.
(178) MCLEAN, F. C. AND A. B. HASTINGS. J. Biol. Chem. **108:** 285, 1935.
(179) MEYER, G. H. Lehrbuch der Anatomie. Leipzig, Engelmann, 1861. Cited by MAYEDA.
(180) MEYER, F. Verhandl. Cong. inn. Med. **19:** 452, 1901.
(181) MEYER, K., E. M. SMYTH AND M. H. DAWSON. J. Biol. Chem. **128:** 319, 1939.
(182) MOND, R. AND F. HOFFMANN. Pflüger's Arch. **221:** 460, 1929.
(183) VON MOSENGEIL. Arch. f. klin. Chir. **19:** 452, 1876.
(184) MOUCHET, A. Compt. rend. Soc. de biol. **69:** 271, 1910.
(185) MOUCHET, A. Anat. Anz. **38:** 71, 1911.
(186) MOUCHET, A. Compt. rend. Soc. de biol. **70:** 9, 1911.
(187) MÜLLER, W. Biologie der Gelenke. J. A. Barth, Leipzig, 1929.
(188) MÜLLER, W. Deutsch. Ztschr. f. Chir. **218:** 395, 1929.
(189) MÜLLER, W. AND H. J. LAUBER. Beitr. z. klin. Chir. **155:** 39, 1932.
(190) MUNTWYLER, E., C. T. WAY AND E. POMERENE. J. Biol. Chem. **92:** 733, 1931.
(191) MYERS, W. K., C. S. KEEFER AND W. F. HOLMES, JR. J. Clin. Investigation **13:** 767, 1934.
(192) NICOLADONI, C. Wien. med. Jahrbuch **4:** 401, 1873.
(193) NIVEN, J. J. Path. and Bact. **34:** 307, 1931.
(194) NUSSBAUM, A. Deutsch. med. Wchnschr. **49:** 849, 1923.
(195) NUSSBAUM, A. Virchow's Arch. **270:** 309, 1928.
(196) ODA, M. Mitt. a. d. Med. Akademie in Kioto **14:** 677, 1935.
(197) OGSTON, A. J. Anat. and Physiol. **10:** 49, 1875.
(198) OGSTON, A. J. Anat. and Physiol. **12:** 503, 1878.
(199) OPPENHEIMER, C. Handbuch d. Biochemie. Ergänzungsband II, 1934.
(200) OSCHKADEROW, W. T. Anat. Anz. **66:** 377, 1929.
(201) PAAS, H. R. Arch. f. klin. Chir. **188:** 1, 1937.
(202) PAYR, E. Deutsch. med. Wchnschr. **44:** 817, 1918.
(203) PESCATORI, F. La Chirurgia degli organi di Movimento **14:** 451, 1930.
(204) PETERS, J. P. Body water. Thomas, Springfield, 1935.
(205) PETERSEN, H. Histologie und mikroskopische Anatomie. 3. Abschnitt Munich, p. 257, 1924.
(206) PODKAMINSKY, N. A. Arch. Klin. Chir. **165:** 383, 1931.
(207) POIRIER. Quoted by FICK.
(208) RAINEY, G. London, Edinburgh and Dublin Philosoph. Magazine **29:** 1846. Cited by HAMMAR.
(209) RAUBER, A. A. Zentralbl. f. d. med. Wissensch. **12:** 305, 1874.
(210) RAUBER, A. A. Leipzig, Engelmann, 1876. Quoted by FICK.
(211) REDFERN, P. Month. J. Med. Sc. **13:** 201, 1851.
(212) REICHERT, K. B. Arch. f. Anat. Physiol. u. wissensch. Med. p. 15, 1849.
(213) RETTERER, E. J. d'Anat. **22:** 256, 1896.
(214) REYHER, C. Deutsch. Ztschr. f. Chir. **3:** 189, 1873.
(215) REYHER, C. J. Anat. and Physiol. **8:** 261, 1874.
(216) RHINELANDER, F. W., G. A. BENNETT AND W. BAUER. J. Clin. Investigation **18:** 1, 1939.

(217) RIMANN, H. Virchow's Arch. **180**: 446, 1905.
(218) ROBERTSON, W. v. B., M. W. ROPES AND W. BAUER. Unpublished.
(219) ROPES, M. W. Am. J. Physiol. **113**: 112, 1935.
(220) ROPES, M. W., G. A. BENNETT AND W. BAUER. J. Clin. Investigation **18**: 351, 1939.
(221) ROPES, M. W., H. C. COGGESHALL, E. C. ROSSMEISL AND W. BAUER. Unpublished.
(222) ROPES, M. W., E. C. ROSSMEISL AND W. BAUER. Unpublished.
(223) ROSENOW, E. C. J. Infect. Dis. **6**: 245, 1909.
(224) ROUVIÈRE, H. Ann. d'Anat. path. **14**: 721, 1937.
(225) ROUX, W. Ges. Abh. **1**: 376, 1887.
(226) ROUX, W. Biol. Zentralbl. **11**: 188, 1891.
(227) RÜDINGER, N. Die Gelenknerven des menschlichen Körpers. Erlangen, Enke, 1857.
(228) RYNEARSON, E. H. J. Bone and Joint Surg. **13**: 127, 1931.
(229) SABRAZÈS, J. AND R. DE GRAILLY. Bull. Soc. Biol. **106**: 1155, 1931.
(230) SALKOWSKI, E. Virchow's Arch. **131**: 304, 1893.
(231) SANTO, E. Anat. Anz. **85**: 223, 1937.
(232) SAPPEY, P. C. Traité d'anatomie descriptive, Delahaye. Paris **1**: 480, 1867.
(233) SCHITTENHELM, A. AND B. EISLER. Ztschr. f. d. ges. exper. Med. **86**: 383, 1933.
(234) SCHMIDT, F. Ztschr. f. d. klin. Chir. **5**: 1, 1875.
(235) SCHMIDT, C. L. A. AND D. M. GREENBERG. Physiol. Rev. **15**: 297, 1935.
(236) SCHNEIDEMÜHL, G. Arch. f. wissensch. u. prakt. Thierh. **10**: 40, 1884.
(237) SCHNEIDER, J. Biochem. Ztschr. **160**: 325, 1925.
(238) SCHRANT, J. M. Der Ursprung des Colloids: nach dem Holländischen von C. E. Weber, Separatabdruck s. e. Cited by HAMMAR.
(239) SCHULIN, K. Arch. f. Anat. u. Entwcklngsgesch. p. 240, 1879.
(240) SCHWEIGGER-SEIDEL, F. Arb. aus. d. Physiol. Anstalt zu Leipzig, p. 150, 1866.
(241) SEELIGER, P. Deutsch. Ztschr. f. Chir. **198**: 11, 1926.
(242) SEGALE, C. Beitr. z. klin. Chir. **81**: 259, 1913.
(243) SEGGEL, R. Deutsch. Ztschr. f. Chir. **75**: 453, 1904.
(244) SHAFFER, M. F. AND G. A. BENNETT. J. Exper. Med. **70**: 293, 1939.
(245) SHANDS, A. R., JR. Arch. Surg. **22**: 137, 1931.
(246) SHDANOW, D. A. Anat. Anz. **69**: 194, 1930.
(247) SIGURDSON, L. A. J. Bone and Joint Surg. **12**: 603, 1930.
(248) SILBERBERG, M. Proc. Soc. Exper. Biol. and Med. **34**: 333, 1936-37.
(249) SILBERBERG, M. AND R. SILBERBERG. Growth **2**: 327, 1938.
(250) SILBERBERG, M. AND R. SILBERBERG. Am. J. Path. **15**: 5, 55, 1939.
(251) SMITH, M. AND J. R. CAMPBELL, JR. Proc. Soc. Exper. Biol. and Med. **26**: 395, 1928-29.
(252) SOUBBOTINE, M. Arch. de Physiol. norm. et path. **7**: 532, s. 2. 1880.
(253) STEINBERG, A. Jahr. Ber. ü. d. Fortsch. d. Anat. u. Physiol., p. 90, 1875.
(254) STRANGEWAYS, T. S. P. Brit. Med. J. **1**: 661, 1920.
(255) SUMITA, M. Arch. f. klin. Chir. **99**: 755, 1912.
(256) TELLO, J. F. Ztschr. f. Anat. u. Entwcklngsgesch. **64**: 348, 1922.

(257) THOMPSON, W. O., P. K. THOMPSON AND M. E. DAILEY. J. Clin. Investigation **5**: 573, 1927–28.
(258) TILLMANNS, H. Arch. f. mikr. Anat. **10**: 401, 1874.
(259) TILLMANNS, H. Arch. f. klin. Chir. **19**: 693, 1875.
(260) TILLMANNS, H. Arch. f. mikr. Anat. **12**: 649, 1876.
(261) TIZZONI, G. Arch. per le Sc. med. **2**: 27, 1877.
(262) TODD, R. B. Cyclopaedia of anatomy and physiology, London **1**: 246, 1836.
(263) TODD, R. B. AND W. BOWMAN. Physiological anatomy and physiology of man. Longmans, London, p. 90, 1845.
Ibid., I, p. 126, 1856.
(264) TOYNBEE, J. Royal Soc. London. Philosophical trans. 131, 159, 1841.
(265) Unpublished.
(266) Unpublished.
(267) VAN DER SLUIYS, J. G. Niederl. Arch. f. Zool. B. 3, 1876–77.
(268) VAN SLYKE, D. D., A. B. HASTINGS, A. HILLER AND J. SENDROY, JR. J. Biol. Chem. **79**: 769, 1928.
(269) VAUBEL, E. Virchow's Arch. **289**: 670, 1933.
(270) VAUBEL, E. J. Exper. Med. **58**: 63, 85, 1933.
(271) WALKER, A. M. J. Biol. Chem. **101**: 239, 1933.
(272) WALKER, A. M. J. Biol. Chem. **101**: 269, 1933.
(273) WALKER, A. M. AND J. A. REISINGER. J. Biol. Chem. **101**: 223, 1933.
(274) WATERFIELD, R. L. J. Physiol. **72**: 110, 1931.
(275) WARREN, C. F., G. A. BENNETT AND W. BAUER. Am. J. Path. **11**: 953, 1935.
(276) WEBER, W. E. AND E. WEBER. Mechanik der menschlichen Gehwerkzeuge. Göttingen, Dietrich, 1836.
(277) WEECH, A. A., E. GOETTSCH AND E. B. REEVES. J. Clin. Investigation **12**: 1021, 1933.
(278) WEICHSELBAUM, A. Wien. Sitzungsber. 1877, Jahrg. 75, Abt. III, 199.
(279) WELLS, H. S. Am. J. Physiol. **101**: 421, 1932.
(280) WINSLOW, J. B. Expos. anat. structurae corporis humani. 1753. Quoted by MÜLLER.
(281) WINTER, W. Biochem. Ztschr. **246**: 10, 1932.
(282) WOLCOTT, W. E. J. Bone and Joint Surg. **9**: 67, 1927.
(283) WOLLENBERG, G. A. Arch. f. Orthopädie **7**: 226, 1909.
(284) ZALEWSKI, A. Travaux de l'Institut d'Anatomie **4**: 1, 1928.
(285) ZELLER, J. W., E. G. L. BYWATERS AND W. BAUER. Unpublished.